Development and application of cell-based bioassays for the evaluation of AhR-mediated effects on human health

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DEVELOPMENT AND APPLICATION OF CELL -BASED BIOASSAYS FOR THE EVALUATION OF AHR -MEDIATED EFFECTS ON HUMAN HEALTH

CLÉMENCE BUDIN

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The design of the cover is inspired by the iceberg analogy, which is sometimes used in Toxicology to represent the vast chemical space. In this illustration by Pauline Boué, the tip of the iceberg represents the small fraction of known chemicals whereas its immerged part represents the hidden, unknown and unseen ones.

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VRIJE UNIVERSITEIT

Development and application of cell-based bioassays for the evaluation of AhR-mediated effects on human health

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List of abbreviations

%CV:	Coefficient of variation
$\%TCDD_{max}$:	2,3,7,8-TCDD max efficiency
$\%VC_R$:	Coefficient of reproducibility
AFB1:	Aflatoxin B1
AFBO:	Exo-AFB1-8, 9-epoxide
AhR:	Aryl hydrocarbon receptor
AhRR:	Aryl hydrocarbon receptor repressor
ARNT:	Aryl hydrocarbon nuclear translocator
B[a]P:	Benzo[a]pyrene
BEQ:	Bioanalytical toxicity equivalent
BEQ_T:	Theoretical bioanalytical toxicity equivalent
CALUX:	Chemically activated luciferase expression
CYP450s:	Cytochrome P450 enzymes
CYP1A1	Cytochrome P450 1A1
DCC-FCS:	Dextran-coated charcoal-stripped foetal calf serum
DecaBDE:	Decabromodiphenyl ether
DMSO:	Dimethyl sulfoxide
DR CALUX:	Dioxin responsive CALUX
DRE:	Dioxin responsive element
DR_{hp} CALUX:	DR high-performance CALUX
DSB :	Double strand break
EC_{50}:	Half-maximal effect concentration
(US) EPA:	(US) environment protection agency
EROD:	7-ethoxyresorufin-O-deethylase
ESCHER:	European Scientific Committee on Health and Environmental Risks
FCS:	Foetal calf serum

GC-HRMS:	Capillary gas chromatography- high resolution mass spectrometry
HTS:	High-throughput screening
LOEC:	Lowest observed effect concentration
NEAA:	Non-essential amino acids
PAHs:	Polycyclic aromatic hydrocarbons
PBDD/Fs:	Polybrominated dibenzo-p-dioxins and dibenzofurans
PBDEs:	Polybrominated diphenyl ethers
PC_5:	5% 2,3,7,8-TCDD-equivalent concentration
PC_{50}:	50% 2,3,7,8-TCDD-equivalent concentration
PCBs:	Polychlorinated biphenyls
PCDD/Fs:	Polychlorinated dibenzo-p-dioxins and dibenzofurans
PHAHs:	Polyhalogenated aromatic hydrocarbons
R^2:	Correlation coefficient
REP:	Relative potency value
RIVM:	Dutch National Institute for Public Health and the Environment
RLU:	Relative light units
ROS:	Reactive oxygen species
RS :	Replication stress
RT-qPCR:	Reverse transcription – quantitative polymerase chain reaction
TDI:	Tolerable daily intake
TEF:	Toxic equivalency factor
TEQ:	Toxic equivalency
TWI:	Tolerable weekly intake
VerA:	Versicolorin A
WHO:	World Health Organization
WHO-TEF:	World Health Organization-toxic equivalency factors

Chapter 1

General Introduction

1. Background to the study

Throughout evolution, animals, including humans have developed a sophisticated network of enzymes, receptors, transporters, and repair mechanisms acting in concert to recognize and defend organisms from the adverse effects of exogenous chemicals. However, when such chemicals are present at sufficient levels, these mechanisms can be challenged. This can result in the disruption of normal physiology through different mechanisms, e.g. by toxicity to cells, mimicking of hormonal effects or affecting the integrity of proteins or the genome, and possibly lead to short- or long-term adverse health outcomes or impaired development (Birnbaum and Fenton, 2003; Colborn, 1992; Kavlock et al., 1996; Safe, 2000).

Exposure to exogenous chemicals typically occurs as mixtures through various routes, like the ingestion of contaminated food and water or inhalation (Fiedler et al., 1990; Rice Carol et al., 2003; Takigami et al., 2009; Tue et al., 2013). The mixtures of chemicals humans are exposed to are very complex by definition and this complexity complicates hazard assessment as well as the establishment of causative relationships between exposure and observed effects (Hernández and Tsatsakis, 2017; Teuschler et al., 2002). Current challenges in toxicology are how to make comprehensive analyses of the toxic chemicals within these mixtures, to determine the total toxic activity associated with exposure to these mixtures, as well as establishing associations between chemical exposures and observed adverse health effects.

Using analytical chemistry-based methods it is possible to determine the concentration of a selected number of chemicals with known toxicity in a mixture. These methods have advanced enormously over the last decades, but they still focus on only a limited number of chemicals considering the vast chemical space. Moreover, they overlook unknown contaminants, and compounds with unknown toxicity and lack the ability to assess biological relevance which is nonetheless essential to assess the toxicity of mixtures (Hernández and Tsatsakis, 2017; Teuschler et al., 2002; Tsatsakis et al., 2016).

To address this issue, based on knowledge on the biological mode of action of toxic chemicals, bioassays have been developed that can be used for comprehensive analyses of the extent of interaction of mixtures with toxicologically relevant biological pathways. The use of these mechanism-based bioassays is becoming increasingly more accepted and they have been proven to be suitable for chemical safety assessment, quantification of mixture effects and assisting in the identification of novel contaminants to which humans are exposed to (Besselink et al., 2017; Piersma et al., 2013; Tue et al., 2010; van der Burg et al., 2015).

There is a need to expand the toxicological knowledge regarding the effect of chemical mixtures, to achieve a better comprehension of chemically induced adverse health effects as well as the implementation of strategies and regulations to protect individuals. Therefore, (human) relevant and reliable tools that can be efficiently applied to characterize the potentially toxic effects of single chemicals, as well as complex mixtures

on human health, would greatly contribute to better comprehend the risks related to exposure. The research described in this thesis will focus on the aryl hydrocarbon receptor (AhR), a major and relevant biological target of different classes of environmental contaminants, and the development and application of *in vitro* mechanism-based bioassays to investigate the effect of chemicals and mixtures of chemicals on the AhR in relation to human adverse health effects.

2. The aryl hydrocarbon receptor, roles, and ligands

2.1. Generalities and roles

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor protein from the basic helix-loop-helix/Per-Arnt-Sim family (b-HLH-PAS; Larigot et al., 2018). The AhR is best known for its role in mediating the toxic effects of environmental contaminants such as the polyhalogenated aromatic hydrocarbons (PHAHs, see 2.2. and 3.). As such, the AhR transduction pathway after ligand-binding activation, described in Figure 1, is comparable to that of nuclear receptors such as the steroid receptors. The translocation of the Ah-receptor into the nucleus is initiated by ligand-binding, activation of the receptor and recruitment of co-factors such as the aryl hydrocarbon nuclear translocator (ARNT) and chaperone proteins. In the nucleus, the AhR-ARNT complex binds to upstream regions of genes containing AhR-responsive elements, also known as dioxin-responsive elements (DREs) and induces the expression of AhR target genes.

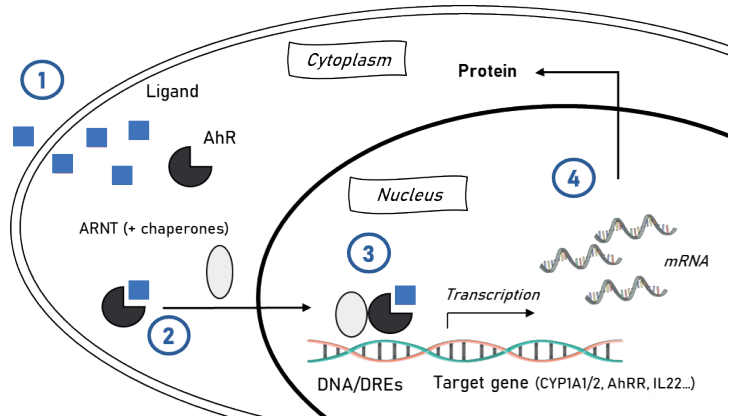

Figure 1. *Simplified scheme of the activation of the AhR pathway by AhR ligands. Ligands entering the cell (1) bind to the Ah-receptor; (2) activating the latter and initiating the recruitment of the AhR Nuclear Translocator (ARNT) and translocation of the ligand-AhR/ARNT complex from the cytoplasm to the nucleus (3). The complex then binds to specific dioxin responsive elements (DREs), DNA regions present in the promoter regions of AhR target genes and increases their expression (4).*

The most studied target genes of the AhR are the cytochrome p450-1A1, 1A2 and 1B1 (CYP1A1/2, CYP1B1) enzymes involved in the metabolism of exogenous chemicals. The induction of the expression of these enzymes, particularly CYP1A1, is often used as a biomarker of AhR activation and is seen as an early warning of toxicity. The toxicological role of the AhR was the first and nowadays the most described role of the receptor. However, research involving AhR-null laboratory animals demonstrated that the function of the receptor is wider than mediation of PHAHs' toxic effects as it plays an important role in, e.g., the control of the cell-cycle, cell-differentiation, modulation of the immune system and organ development (Esser and Rannug, 2015; Safe et al., 2013).

Because of the functions it fulfils, the presence of the AhR is generally accepted to be conserved throughout the animal kingdom. Nonetheless, structural, and functional species-specific differences exist. These differences can be reflected by different ligand-binding affinities, specificities and differential responses after exposure to specific ligands (Connor and Aylward, 2006; Denison et al., 2002; Denison and Faber, 2017; Hahn et al., 2017). However, their origin and implications are not fully understood yet and remain to be further investigated.

2.2. Ligands of the AhR

Different chemicals are known to be ligands of the AhR. The most studied group are the polyhalogenated aromatic hydrocarbons (PHAHs), which consists of classes of chemicals that are considered environmental contaminants: polyhalogenated dibenzo-p-dioxins and furans (dioxins), polyhalogenated biphenyls like PCBs and polycyclic aromatic hydrocarbons (PAHs; Denison and Nagy, 2003). Dioxins are high-affinity ligands of the receptor (pM to nM range) whereas PAHs generally display a weaker affinity to the AhR than dioxins (nM to μM) which may to a large extent be explained by the fact that they are more easily metabolised by organisms and thus, only transiently activate the AhR whereas dioxins are persistent activators of the receptor (Pieterse et al., 2013). Species-specific differences in dioxins' potency to activate the AhR have been reported in which the human receptor appeared much less sensitive than e.g. the rodent AhRs (Connor and Aylward, 2006). However, in all species, the activation of the AhR by PHAHs results in a toxic response, which will be further discussed in section 3. Additionally, the AhR mediates the activation of certain pro-carcinogenic PAHs that are both ligands of the receptor and substrate of CYP1A1.

Several exogenous chemicals other than PHAHs have been demonstrated or suggested to be able to activate the AhR albeit showing a lower receptor binding affinity as compared to PHAHs (Denison and Nagy, 2003; Murray and Perdew, 2020). These chemicals with highly diverse structures sometimes greatly differing from PHAHs include natural dietary chemicals, microbial- and fungal metabolites (Arenas-Huertero et al., 2019; Denison and Nagy, 2003; Murray and Perdew, 2020). Unlike PHAHs, these chemicals do not always produce a toxic response, and species-differences in responses and sensitivities have been reported (Flaveny et al., 2009; Hubbard et al., 2015). Over the last decade, studies

also proposed the existence of an interaction between mycotoxins, major contaminants of food commodities produced by fungi, and the AhR pathway (Arenas-Huertero et al., 2019; Ayed-Boussema et al., 2012). This interaction is suggested by the induction of AhR target-genes, particularly phase I metabolism enzymes (e.g. CYP1A1/2) which catalyse the epoxidation of certain mycotoxins into DNA-reactive intermediates. However, the evidence of direct interaction via ligand-binding remains to be clearly demonstrated (Arenas-Huertero et al., 2019). As the AhR is known to mediate the activation of pro-carcinogenic PAHs it is of interest to determine if such a mechanism may also occur in the case of pro-carcinogenic mycotoxins, such as the aflatoxins. Finally, several endogenous AhR ligands have been described of which the physiological role has not been fully elucidated, but are likely important in physiological processes such as immune system functioning, haemopoietic stem cell renewal and differentiation (Angelos et al., 2017; Larigot et al., 2018).

Overall, the AhR can be activated by an array of ligands wider and more diverse than initially thought. Moreover, species differences in sensitivity, specificity and responses have been reported for different AhR ligands which further emphasize that the receptor plays a role in physiology aside its toxicological functions. From a human-health based hazard assessment perspective, it is of interest to clarify if AhR-related species-differences can hamper the predictability of suspected effects in humans when toxicity data are generated in non-human models. This is particularly important for contaminants which humans are susceptible to and exposed to, such as dioxins, PAHs and mycotoxins.

3. Health effects of toxic AhR ligands

The ligand-dependent activation of the AhR results in an extensive and diverse spectrum of effects which are to date not completely understood (Denison and Faber, 2017). However, it is accepted that while diverse ligands can activate the AhR, most of them do not produce the same toxicological and biological effects as PHAHs. The toxicity of PHAHs, particularly dioxins, was demonstrated from *in vivo* and *in vitro* toxicological studies which established the induction of specific toxic responses in different species (e.g. cancer, immune suppression, reproductive- and developmental toxicity), referred to as "dioxin-like" toxicity. (Birnbaum, 1994; Poland and Knutson, 1982; Safe, 1986). In the case of PAHs, although they have been demonstrated to be ligands of the AhR and causing toxic *in-vivo* adverse effects, the term "dioxin-like" toxicity does not strictly apply as they are not persistent ligands of the Ah-receptor and produce a range of effects which differ from those elicited by dioxins (Denison and Heath-Pagliuso, 1998; van den Berg et al., 1998).

3.1. Dioxins

Dioxins are unwanted by-products from anthropogenic activities (e.g. waste incineration, paper bleaching, metal production). Over 90% of human exposure to dioxins occurs through the ingestion of contaminated food, mainly meat and dairy products, fish, and shellfish (WHO). In humans, acute exposure to small amounts (μg-range) of highly toxic

dioxins results in characteristic skin lesions known as chloracne (Sorg et al., 2009) whereas equivalent doses are lethal for laboratory animals such as guinea pigs and mice (Birnbaum, 1994). Thus, from a comparison of toxicological data in experimental animals and observed by accidental exposure in humans, it is concluded that humans are relatively insensitive to dioxins. However, the persistence of dioxins in the human body, particularly in fatty tissues, is key to their long-term and wide range of adverse effects (Brouwer et al., 1995; Schecter et al., 2006; Watanabe et al., 1999).

The long-term adverse effects of dioxins on human health have been demonstrated in a number of epidemiological studies (Schecter, 2013). Some well-known examples are the studies following accidental exposure to dioxins in the Seveso incident (Bertazzi et al., 1998) and the spraying of dioxin-contaminated Agent Orange during the Vietnam war (Pavuk et al., 2003; Schecter et al., 2006). Epidemiological studies have also demonstrated that the early stages of life (prenatal and postnatal) are the most sensitive periods for exposure and the disruption of AhR signalling by dioxins at this stage of life may result in visible and invisible birth defects such as cognitive-, immune- and endocrine adversities (Birnbaum L S, 1995; Brouwer et al., 1995; Eskenazi et al., 2018; Tusscher and Koppe, 2004).

3.2. Polycyclic aromatic hydrocarbons

PAHs are a large and heterogeneous class of pervasive contaminants released from combustion processes e.g. of wood, coal, biofuels and cigarette smoking (Choi et al., 2010). Inhalation represents the main route of exposure to PAHs although they can also contaminate food (e.g. cooked/smoked foods). In the case of PAHs, acute and short-term exposure in laboratory animals and humans results in inflammatory responses, vomiting and diarrhea.

Long-term occupational exposure to PAHs has been associated with cancers, reproductive- and developmental effects (Kim et al., 2013). Most of the knowledge regarding the effects of long-term PAH exposure results from studies where subjects have been occupationally exposed to PAHs e.g. via inhalation in the work environment or cases like direct exposure to cigarette smoke (Kim et al., 2013). However, studies have associated pre- and postnatal exposure to PAHs containing mixtures, such as atmospheric pollution or cigarette smoke, to adverse developmental and neurobehavioral effects in neonates or children (Fowler et al., 2014, 2008; Perera et al., 2005; Perera et al., 2012). This indicates that certain adverse effects may be potentially related to the presence of PAHs in those matrices. Nonetheless, current knowledge on PAH exposure is insufficient to firmly conclude on their long-term effects and the possible role of PAHs as causative chemicals in certain adverse outcomes remains to be firmly established, particularly concerning prenatal exposure.

Life stage-, tissue- and species-specific differences in PHAHs sensitivities exist and it is important to consider these in the evaluation of health effects. However, the early stages of life such as the prenatal and postnatal period are unequivocally the most sensitive

regarding PHAHs exposure. Yet, the knowledge regarding the biological effects of chemical mixtures during human development is limited. An approach to better comprehend the developmental effect of mixtures is to study their effect on key biological pathways through which chemicals can act. In the case of PHAHs, it would be relevant to investigate the effect of mixtures to which developing humans are exposed on the AhR, as it is a major target of PHAHs which also plays an important role during development. However, this implies that it is needed to apply methods that enable studying AhR functioning as a result of exposure to chemical mixtures.

4. Hazard and risk assessment

Regarding the classes of chemicals that will be investigated in this thesis (dioxins, PAHs and mycotoxins), regulations are in force to limit and/or monitor exposure to these chemicals. In the case of dioxins and PAHs, approaches have been proposed to better assess the risk related to mixtures.

4.1. Dioxins

Given the experimental and epidemiological evidence that dioxins are highly toxic chemicals, great efforts have been made to develop approaches and strategies to monitor and regulate their presence in the environment and food. This led, amongst others, to the development and application of toxic equivalency factors (TEFs) for dioxin-like chemicals regulating the presence of 17 polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs), and 12 dioxin-like PCBs (van den Berg et al., 1998). The TEF values are based on a range of short term and in particular long-term adverse health effects observed in experimental animals and in humans, which are mostly based on the AhR-mediated mode of action of these compounds and defines the toxic potency of a specific congener relative to 2,3,7,8-TCDD (the most toxic congener) which was assigned a TEF=1. Under the WHO-TEF scheme, the total toxic equivalency (TEQ) of a PCDD/Fs and PCBs mixture is calculated by multiplying the concentration of each congener determined via the analytical-chemical method (i.e. gas-chromatography coupled to mass-spectrometry, GC-MS) by its corresponding TEF and then summing up the results to obtain a single value expressed in e.g., pg TEQ per g of sample. The WHO-TEF scheme has been used to estimate dioxin equivalence in (a)biotic samples and, despite some limitations, has been proven to be useful and valuable to monitor dioxin levels, leading to better control/evaluation of the environmental and dietary exposure levels. Alternatively, based on the AhR-mediated mode of action of dioxin-like chemicals, cell-based methods have been developed for integrated toxicological hazard assessment and quantification of PCDD/Fs and dioxin-like PCBs mixtures (see section 5.2.).

Apart from chlorinated dioxins, there is now evidence accumulating that brominated dioxins (PBDD/Fs) can be found in diverse biotic and abiotic matrices (Choi et al., 2003; Pajurek et al., 2019; Suzuki et al., 2017; Tue et al., 2013) and concerns arose with respect to their toxicological and environmental significance in comparison to their chlorinated counterparts (Birnbaum et al., 2003; Fernandes and Falandysz, 2020; van den Berg et al.,

2013). As brominated dioxins are nowadays suspected to significantly contribute to the total daily background exposure to dioxin-like chemicals, it was recently suggested to include them in the WHO-TEF scheme using interim TEF values based on the TEF values of their chlorinated counterparts (van den Berg et al., 2013). There is, however, a limited analytical capacity to analyse PBDD/Fs (Hagberg, 2009) which is likely one of the main reasons that they are rarely included in monitoring studies to this date.

4.2. Polycyclic aromatic hydrocarbons

PAHs are not included in the TEF-scheme as they do not meet basic inclusion criteria such as high persistency and they do not elicit dioxin-like toxicity (Nisbet and LaGoy, 1992; Van den Berg et al., 1998). Furthermore, PAHs are a large group of chemicals which, unlike dioxins, are relatively heterogeneous with widely varying physical and chemical properties making their chemically targeted analytical measurement extremely laborious. This is the reason why a pragmatic list of 16 prioritized PAHs has been issued by the US Environment Protection Agency (EPA) to facilitate monitoring and analysis of PAHs (Keith, 2015). Unlike in the WHO-TEF scheme, this list is not associated with a congener-specific toxicity factor and therefore cannot provide any toxicological information about the mixture of PAHs present in a matrix. Yet, it has been suggested to use a crude version of the TEF approach described above, based on the carcinogenic potential of the EPA-16 PAHs to give a rough estimation of the carcinogenic potency of a mixture when using analytical-chemistry (Nisbet and LaGoy, 1992). In a later study using an *in vitro* reporter gene assay specifically targeting PAHs (PAH CALUX), the carcinogenic potency of the EPA-16 PAHs appeared to correspond with their ability to activate the AhR, emphasizing the role of the AhR in PAH-mediated carcinogenicity and overall toxicity (Pieterse et al., 2013). More recently, Long et al., also found evidence for dose-additivity as a good model to predict the genotoxicity of mixtures of PAHs in a transgenic mouse model (Long et al., 2017).

4.3. Mycotoxins

The hazard evaluation and risk assessment of mycotoxins are based on the quantification of single fungal metabolites (e.g. aflatoxin B1, deoxynivalenol, T-2 toxin). No methodology or approach has yet been developed which allows making a comprehensive analysis of the toxicological effects of mycotoxin mixtures (Assunção et al., 2016; de Nijs et al., 2016). However, interactions may occur between individual mycotoxins within one chemical class, and/or between different chemical classes. For example, in the case of aflatoxins (Theumer et al., 2018), there is limited data available regarding the toxicity of aflatoxins' biosynthetic precursors alone or in combination. Nevertheless, it is anticipated that mycotoxins from the same chemical class may share similar toxicity profiles and modes of action. This suggests that chemically related mycotoxins may exert combined additive effects on certain quantifiable molecular events.

5. Bioassays for the analysis of AhR-mediated activity; principle and applications

5.1. Principle

Bioassays are organism- or cell-based methods that can be applied to study the effect of chemicals on intact or isolated biological systems. In mechanism-based bioassays, the readout is coupled to events such as receptor or pathway activation. In the case of the AhR, bioassays are based on biochemical endpoints of the AhR pathway such as AhR transactivation, which can be quantified using, e.g., luciferase-expressing reporter cell lines. Luciferase reporters are cell lines stably (or transiently) transfected with a plasmid containing the luciferase gene under transcriptional control of artificially introduced dioxin responsive elements (DREs) which confers to the reporter cell line its selective and specific AhR-responsiveness. The principle of luciferase reporter gene assays is described in Figure 2, showing the example of an AhR-mediated assay. Briefly, exposure of reporter cells to AhR ligands results in receptor activation and subsequent dose-dependent luciferase transcription and synthesis. The amount of luciferase enzyme synthesised is then measured after the addition of its substrate (luciferin) whose oxidation results in quantifiable light emission (luminescence).

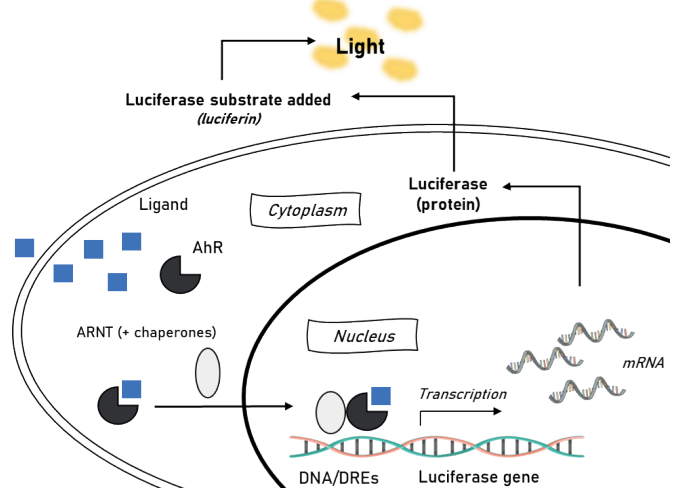

Figure 2. *AhR-mediated mechanism of luciferase gene expression in a reporter gene assay. See text for further explanation.*

5.2. Analysis of AhR-active mixtures

A major application of AhR-mediated bioassays is the detection and quantification of dioxin-like chemicals, by their AhR-mediated responses in samples. Bioassays provide a readout expressed as a total activity-equivalent value, referred to as bioanalytical equivalent (BEQ), representative of the total effect of the mixture on the AhR whereas the analytical chemistry-based TEQ described in section 4.1. only provides information about

congeners for which standards (and methods) are available thus bypassing unknown or unexpected chemicals which can interact with the AhR. These bioassays were developed using cell lines derived from very dioxin-sensitive species (e.g. rodent), which is key to their ability to efficiently detect and quantify low amounts of dioxin-like activity.

Coupled to selective sample clean-up procedures, methodologies such as the Dioxin Responsive chemically activated luciferase expression method (DR CALUX®) have been widely used as an alternative and/or complement to traditional analytical-chemical-based methods for the quantification and regulatory control of dioxins and dioxin-like PCBs in a variety of matrices (e.g. food, feed sediments). AhR-mediated bioassays have also been used in human biomonitoring and epidemiological studies using blood, serum, or breast milk as a proxy to evaluate the levels of exposure to dioxins and dioxin-like PCBs in populations (Croes et al., 2013; Iszatt et al., 2016; Müller et al., 2019; Porpora Maria Grazia et al., 2009; Tue et al., 2014; Vinggaard et al., 2021). In some studies, bioassay analysis was compared to analytically determined TEQ and a good correlation was found, supporting the relevance of the bioassays for biomonitoring and epidemiological purposes (Vinggaard et al., 2021). Although, multiple studies have shown that it is possible to analyse AhR activity in human samples it remains challenging. In particular because of the sometimes-limited amount of plasma/tissue available for this type of analysis, e.g., in studies focusing on prenatal and postnatal periods. Therefore, it may be necessary to develop bioassays that are even more sensitive than those available now, to make such analysis possible and facilitate human biomonitoring, consequently enabling us to gain knowledge regarding human exposure and investigate the associations between chemical exposures and observed adverse health effects.

5.3. Hazard assessment of individual chemicals

Reporter gene assays are also used in more straightforward applications. As an example, AhR-responsive bioassays can be used for the identification of ligands (Hinger et al., 2011; Long et al., 2003; Takeuchi et al., 2008) and the determination of relative potencies for dioxin-like chemicals (Behnisch et al., 2003; Jones and Anderson, 1999; Olsman et al., 2007). Reporter gene assays are also compatible with high-throughput screening (HTS) systems which enables the activity screening of large compound libraries (van der Burg et al., 2015). HTS is a recent innovation which is a primary tool for drug discovery that expanded to the field of toxicology as a complement to traditional toxicity testing (van der Burg et al., 2013; Dix et al., 2007). Nowadays, reporter gene assays and other *in vitro* bioassays are also globally gaining regulatory acceptance regarding their use for chemical hazard assessment (Besselink et al., 2017; Hartung, 2017). In contrast to the quantification of dioxin-like activity in samples, where it is preferable to use cell lines as sensitive as possible, species-specific bioassays may be more appropriate for hazard assessment of individual chemicals. However, in the case of the AhR, since the focus has been on developing sensitive quantitative bioassays, current testing strategies lack availability of efficient human-based bioassays. Considering the existence of AhR-related species-differences, the use of bioassays based on non-human organisms to predict

effects in humans may represent a weakness to human hazard assessment. Hence, it is important to develop and make available human-relevant *in vitro* methods to investigate the effect of chemicals on the AhR in a human cellular context to enhance hazard assessment. Moreover, as human exposure to chemicals occurs as mixtures, it would be advantageous for these methods to further be applicable for the analysis of complex mixtures of AhR-active chemicals on the human AhR.

6. Scope & outline of the thesis

This thesis aims to develop and apply novel *in vitro* bioassays to evaluate the effect of chemicals and mixtures of chemicals on the AhR with the objective to improve human relevance and optimize the interpretability of bioassay results towards human exposure. The research presented focused on the interaction of different classes of chemicals (dioxins, PAHs and mycotoxins) with the AhR and its public-health relevance. Species-specific differences regarding AhR-signalling in terms of hazard prediction were also discussed because of their potential implications for human health-based hazard assessment.

In **chapter 2**, a human-based AhR luciferase reporter gene assay was developed to provide a human cellular-context needed for the subsequent studies. In this chapter, we applied the newly developed human liver cell-based DR_{human} CALUX bioassay to determine relative potency values for PCDD/Fs and PBDD/Fs. The values were compared to values obtained in the rodent-based DR CALUX bioassay and the WHO-TEF values for hazard assessment. **Chapter 3** describes an application of the DR_{human} CALUX bioassay for the determination of dioxin-like activity in consumer goods, such as plastic toys. An analytical chemistry-based measurement was compared to the activity *in vitro* determined by the DR CALUX and its human variant, and a first approximation of human hazard assessment based on the ingestion of contaminated plastic from toys by children was performed. **Chapter 4** describes a second AhR-mediated bioassay, a high performance (hp) variant of the rodent-based DR CALUX bioassay with improved sensitivity, the DR_{hp} CALUX. In this study, we showed that the bioassay is suitable for highly sensitive detection and quantification of low levels of PAH-mediated AhR activity in human samples. Hence, the DR_{hp} CALUX was applied to quantify the effect of exposure to PAHs via maternal smoking on placental and foetal hepatic PAH-based AhR activity. **Chapter 5** describes a study in which *in vitro* bioassays, including the DR_{human} CALUX, were applied to individual and combined hepatotoxicity of carcinogenic mycotoxins aflatoxin B1 and a main fungal precursor, versicolorin A. This study sheds a light on a previously unexplored role of the AhR concerning the carcinogenicity of aflatoxins and discusses hazard assessment related to aflatoxin mixtures. Finally, **Chapter 6** summarizes the most important findings of the studies in this thesis, discusses their relevance for hazard assessment, and presents an outlook for future work.

7. Involvement in the Horizon 2020 ITN ProtectED project

The research presented in this thesis was conducted between 2017 and 2020 within the EU Horizon 2020 research and innovation program under the Marie Sklodowska-Curie ITN ProtectED (PROTECTion against Endocrine Disruptors: detection, mixtures, health effects, risk assessment and communication; grant agreement No. 722635). The work presented in chapter 4 & 5 is the result of collaborations from BioDetection Systems B.V. with partners from the ProtectED network, the University of Aberdeen (chapter 4) and the French National Research Institute for Agriculture, Food and the Environment (INRAe, chapter 5). The primary aim of the ProtectED project was to develop innovative and improved analysis capabilities for the risk assessment (and communication) of the impact of endocrine disruptors and their mixtures on human health and the environment.

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Chapter 2

Induction of AhR transactivation by PBDD/Fs and PCDD/Fs using a novel human-relevant, high-throughput DR_{human} CALUX reporter gene assay

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Abstract

Polychlorinated dioxins and dibenzofurans (PCDD/Fs) are highly toxic contaminants that are strictly regulated and monitored in the environment and food to reduce human exposure. Recently, the increasing occurrence of polybrominated dioxins and dibenzofurans (PBDD/Fs) in the environment is raising concerns about the impact on human health by the combined exposure to chlorinated and brominated analogues of dioxins. Toxicological properties of PBDD/Fs relative to PCDD/Fs have not been firmly established, and brominated dioxins are not included in routine monitoring programs. In this study, we set out to determine human-relevant congener-specific potency values for a range of brominated and chlorinated dioxin congeners, based on their aryl hydrocarbon receptor (AhR)-mediated mode of toxic action. Transactivation of the AhR was measured using dioxin-responsive (DR) CALUX reporter gene assays. Because of known species-differences in dioxin-mediated toxicity, we developed and used a HepG2 human liver cell-based DR_{human} CALUX bioassay that is a variant of the rodent-based DR CALUX. The bioassay was found to be highly inducible and stable, with low variations between independent measurements. Using both DR CALUX bioassays in an automated high-throughput mode we found that overall PBDD/Fs were as potent as PCDD/Fs in inducing AhR transactivation, but congener-specific differences were observed. We also observed species-specific differences in sensitivity and potency when comparing DR_{human} REP values to those obtained in the rat-based DR CALUX. Finally, we observed significant differences between WHO-TEF values and DR human REP values, suggesting that actual WHO-TEF values may underestimate the hazards associated with exposure of humans to dioxins.

1. Introduction

Chlorinated dioxins are ubiquitous and persistent environmental contaminants that accumulate in the food chain with detrimental effects on human and ecological health (Watanabe et al., 1999; Birnbaum, 1995). Polychlorinated dioxins and dibenzofurans (PCDD/Fs) are strictly regulated and monitored in a variety of matrices such as food, sediment, and human material (Barone et al., 2019; Besselink et al., 2004; Warner et al., 2019). The standard analytical method for the quantification of PCDD/Fs is Gas Chromatography coupled to High-Resolution Mass Spectrometry (GC-HRMS). Concentrations of PCDD/F congeners measured by GC-HRMS are then transformed into toxicologically relevant information using the World Health Organization (WHO-TEF) scheme of toxic equivalency factors (TEFs) to calculate the toxic equivalency (TEQ) which represents the total dioxin-like toxicological activity of the mixture. The TEQ is calculated by multiplying the concentration of each congener by its corresponding TEF and then summing up the results to obtain a value expressed in e.g., pg TEQ per g of sample. The TEF values are consensus factors established from a database of relative potency values (REP) that were determined by in-vivo toxicity studies for each of 17 PCDD/Fs, using mostly rodent models (Haws et al., 2006; Van den Berg et al., 2006).

Most if not all the toxicity of PCDD/Fs is mediated via binding to and transactivation of the dioxin receptor, also known as aryl hydrocarbon receptor (AhR). Based on this property, AhR-driven cell-based methods, such as DR CALUX®, have been developed as an alternative to gas chromatography-based methods for the determination of the toxic potency of PCDD/Fs mixtures in e.g. food, feed and human matrices (Besselink et al., 2004; Gizzi et al., 2005; van Leeuwen et al., 2007). In that case, the activity measured is expressed as picograms bioanalytical toxicity equivalents (BEQ) per gram which is representative of the total dioxin-like activity of all PCDD/Fs congeners capable to transactivate the AhR. Cell-based assays have the advantage to be responsive to any chemical and congener capable of interacting with the AhR other than the 17 regulated PCDD/Fs (and 12 dioxin-like PCBs). Additionally, a combination of AhR-responsive bioassays and GC-HRMS may facilitate the identification of unexpected or unknown dioxin-like contaminants and contamination rapidly and cost-effectively.

More recently, the wide-spread use of brominated flame retardants has raised concerns regarding their presence in the environment, including the associated presence of potentially toxic polybrominated dibenzo-p-dioxins and dibenzofurans (PBDD/Fs) (Choi et al., 2003; Pajurek et al., 2019; Tue et al., 2013). Because PBDD/Fs have chemical and toxicological properties that are comparable to those of the highly toxic polychlorinated dioxins and dibenzofurans (PCDD/Fs), their toxicity and distribution in the environment are of concern (Behnisch et al., 2003; Birnbaum et al., 2003; van den Berg et al., 2013). Human exposure to PBDD/Fs can occur via environmental sources but also indoor sources (e.g. house dust) and may represent a significant additional contribution to the daily human background exposure to dioxins and dioxin-like compounds (Takigami et al., 2009; Tue et al., 2013; van den Berg et al., 2013).

In comparison to the extensive toxicological database for PCDD/Fs, the PBDD/Fs are much less studied, and their toxic potencies have not been firmly established. Nevertheless, temporarily TEF values have been suggested for the 17 PBDD/F congeners corresponding to the potency values of their chlorinated counterpart pending availability of more toxicological data regarding PBDD/F-congener-specific potencies (van den Berg et al., 2013). This, and several analytical challenges have hampered their routine monitoring (Hagberg, 2009) and at present, PBDD/Fs are not included, yet in the WHO-TEF system for the determination of toxicity equivalents (TEQ) of dioxin mixtures during monitoring and regulatory control studies.

To improve this situation, more insight into the toxic potency of PBDD/Fs relative to PCDD/Fs is essential. Because their chemical structure is very similar to that of chlorinated dioxins, PBDD/Fs are also capable to interact with the AhR in a ligand-dependent manner, which is key to their ability to trigger a dioxin-like mechanism of toxicity. Dioxin responsive reporter cell lines, such as DR CALUX also respond to PBDD/F congeners and are thus highly suitable to determine congener-dependent TEF values and are suitable for routine monitoring of total dioxin TEQ content including the PBDD/Fs. Although in-vivo congener-specific relative potency values are given priority for the establishment of TEFs, *in vitro* REP values are proven to be a good proxy of AhR-mediated in-vivo effects of dioxins (Mason et al., 1987a, 1987b) and are relevant to use as a basis for human hazard assessment of PBDD/Fs.

Several studies have reported REP values for a subset of PBDD/Fs in different rodent-based *in vitro* models (Behnisch et al., 2003; Olsman et al., 2007; Suzuki et al., 2017). However, it is known that rodent and human AhR can differ in terms of sensitivity towards dioxins (Aarts et al., 1995; Long et al., 2003) and the induction potency of human AhR transcriptional activity by PBDD/Fs remains uncertain. To investigate these species-differences and its potential consequence on hazard assessment, it is important to consider REPs values determined from comparable models in terms of reporter construct, type of readout and further methodological details. Additionally, the limited available literature using human models does not allow a direct and efficient comparison with their PCDD/Fs analogues, which is nonetheless necessary to evaluate possible differences in potencies between the chlorinated and brominated versions of dioxins and furans (Olsman et al., 2007; Organtini et al., 2017; Wall et al., 2015).

To this end, we developed a human cell-based reporter gene assay, using the HepG2 cell line, which is a variant of the rodent-based DR CALUX (Aarts et al., 1995) that displays a similar dioxin-responsive reporter construction and method of analysis. In this study, we first describe the development of a stable human reporter gene cell line and studied the performance characteristics of this novel DR_{human} CALUX reporter cell line. After optimization of the bioassay to an automated high-throughput analysis format, we determined congener-specific REP values for several chlorinated and brominated dioxins and furans. By determining human-specific REP values we aimed to assess if brominated dioxins were as potent as their chlorinated counterparts in human cells. Comparison of

those values to the ones obtained in the rodent-based DR CALUX bioassay enabled us to investigate potential species differences. Lastly, we compared DR_{human} CALUX derived REPs to the recommended WHO-TEFs values for PCDD/Fs and PBDD/Fs to finally discuss their relevance for human hazard assessment.

2. Material and methods

2.1. Chemicals

Dimethyl Sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium). Congeners 2,3,7,8-TCDD and 1,2,3,4,7,8-HxBDF were purchased as analytical standards from Cambridge Isotope Laboratories (Andover, MA-USA). Congeners 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF, OCDF, 2,3,7,8-TBDD, 1,2,3,7,8-PeBDD, 1,2,3,4,6,7,8-HpBDD, OBDD, 2,3,7,8-TBDF, 2,3,4,7,8-PeBDF ,2,3,4,6,7,8-HpBDF and OBDF were purchased as analytical standards from Wellington Laboratories (Guelph, Canada). To obtain DMSO stocks of the standard solutions in n-nonane, part of the standard solution was evaporated and replaced by DMSO to obtain a stock solution concentrated to 1E-05M for each dioxin congener (highest analyzed concentration after dilution for the assay = 1E-07M for each congener). The final amount of DMSO in exposure medium did not exceed 1%.

2.2. Cell lines and culturing

Wild type U2OS, HepG2, C2BBe1, HT-29 and HEK293 were obtained from the American type culture collection (Manassas, VA-USA) and were cultured and maintained in DMEM:F-12 (Gibco) medium supplemented with 7.5% foetal calf serum (FCS), 10% non-essential amino acids (NEAA), respectively, and streptomycin (10µg/mL) plus penicillin (10U/mL) antibiotics.

The DR CALUX cell line consists of an H4IIe-based (rat) cell line stably transfected with the pGudLuc1.1 AhR-controlled luciferase reporter plasmid (Murk et al., 1996). DR CALUX cells were cultured in α-MEM (Gibco) medium supplemented with 10% FCS and streptomycin (10µg/mL) plus penicillin (10U/mL) antibiotics.

The DR_{human} CALUX cell line is a human variant of the DR CALUX bioassay and consists of a human HepG2 cell line stably transfected with a pGudLuc1.1 AhR reporter construct insulated with sequences of the chicken hypersensitive site-4 gene (Arumugam et al., 2009). DR_{human} CALUX cells were cultured in DMEM:F-12 (Gibco) medium supplemented with 7.5% FCS, 10% non-essential amino acids (NEAA), respectively, and streptomycin (10µg/mL) plus penicillin (10U/mL) antibiotics.

The assay medium used in the case of pure compound analysis by DR and DR_{human} CALUX consisted of phenol-free DMEM:F-12 (Gibco) medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS), 10% NEAA and streptomycin (10µg/mL) plus

penicillin (10U/mL) antibiotics. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

2.3. Transient transfections

Transient transfections were conducted using the calcium-phosphate DNA precipitation method. On the first day, cells from a continuous culture were resuspended in culture medium after which 0.6 mL of the cell suspension was distributed in 24-wells plates incubated under standardized conditions described earlier. Different plating densities were used for the different cell lines: 20 000 cells per well for U2OS, 80 000 cells per well for HEK293, 50 000 cells per well for HepG2 and C2BBe1. On day two, the transfection mix was prepared, and the cells were transfected. The transfection mix consisted of 1µg of plasmid DNA diluted with 40uL of 2xHBSP buffer to which 40uL of 0.25M $CaCl_2$ was added to form a DNA precipitate (per well). The transfection mix was then added to the cells and the plates were placed back in the incubator for 16 hours. On the third day, the transfection medium was discarded, and the cells washed twice with 100uL of PBS before exposure. The exposure medium was then prepared and 800µL of assay medium was used per well containing 0.1% of 2,3,7,8-TCDD dissolved in DMSO. Exposed cells were placed back in the incubator for 24 hours. Thereafter the medium was discarded, and the cells lysed using Triton lysis buffer before the analysis of luminescence was performed. A volume of 150µL of lysis buffer was added to the well and the plate was placed on a shaker for approximatively 10 minutes. Finally, 30µL of the lysate was transferred to wells of a 96-well plate and luciferase activity was measured using a luminometer (Berthold, Bad Wildbad, Germany) following the addition of a luciferin-containing solution, as described before (Besselink et al., 2004).

2.4. Stable transfections

The stable transfection of HepG2 cells was achieved using the Lipofectamine 2000 method according to the manufacturer's instructions (Invitrogen, CA USA). This method is preferred for stable transfections because this method usually results in higher transfection efficiency and therefore a higher number of antibiotic resisting transfectants. HepG2 wild type cells were transfected with the AhR-responsive reporter plasmid and the geneticin resistance plasmid pSG5-neo (Sonneveld et al., 1998). The geneticin concentration used for antibiotic resistance selection of HepG2 transfectants was determined before the transfection and was equal to 600 µg/mL.

2.5. DR_{human} and DR CALUX analyses

DR_{human} and DR CALUX cells from continuous cultures were resuspended in assay medium to a density of 200,000 or 300,000 cells per mL, respectively. Using an automatic multi-channel pipette, 100uL per well of cell suspension was distributed over a transparent 96-well plate and the plates were placed in an incubator. After 24 hours, sub-confluent cells (70%-95% confluence) were retrieved and the exposure medium was prepared. The exposure medium was prepared manually and consisted of a 9-step dilution series in 1 log unit increments of the compound to be analyzed (dissolved in

DMSO) in the assay medium as previously described. Cells were exposed manually, in triplicate, via the addition of 100uL of exposure medium taking good care not to exceed a maximum addition of 1% DMSO. On each plate, a complete 2,3,7,8-TCDD reference dilution series was incubated in addition to the dilutions of the test compounds. Following a 24 hours incubation under standardized conditions, the medium was discarded, and cells were lysed using a Triton lysis buffer and the plate was mixed on a shaker for approximately 5 minutes. The luciferase activity was measured after the addition of a luciferin-containing solution using a luminometer plate reader (Berthold, Bad Wildbad, Germany).

2.6. CALUX high-throughput analyses

For pure compound analysis, automated CALUX assays were performed (van der Burg et al., 2013). Reporter cells from continuous culture were resuspended in assay medium to a density of 200,000 and 300,000 cells per mL for DR_{human} and DR CALUX respectively. Suspensions of DR and DR_{human} CALUX reporter cells were distributed over a white 384-wells plate using a multi-channel automatic pipette. A volume of 30uL of suspension was distributed per well and the plates were placed in an incubator. After 24 hours, sub-confluent reporter cells (70-95% confluence) were retrieved and the exposure medium was prepared. The exposure medium was prepared using a liquid handling robot (Hamilton Starlet, Hamilton, USA). A 13-step dilution series in 0.5 log unit increments, starting at 1E-05 M of each test compound (dissolved in DMSO) was prepared in the assay medium. The dilutions series were used by the liquid handling robot to expose the cells. All compounds were analyzed at least two times in triplicate and under reproducible conditions. The same exposure medium was used for both DR and DR_{human} CALUX.

On each plate, a complete 13-points 2,3,7,8-TCDD reference dilution series (starting at 1E-08M in the well) was analyzed in quadruplicate and the rest of the plate was used to analyze a 13-points full-concentration range of a maximum of 6 compounds (in triplicate). Following a 24 hours incubation, the cells were lysed using Triton lysis buffer. Ultimately, the luciferase activity was measured using a luminometer as described in section 2.5.

2.7. Data analysis

The software GraphPad Prism was used for dose-response modelling employing a four-parameter nonlinear regression model (1):

$$(Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10\text{^}((\text{LogEC50} - X) \times \text{HillSlope}) \quad (1)$$

Using raw luminescence data (RLU, relative light units), the maximum response of the reference compound 2,3,7,8-TCDD was set at 100% and the response of individual congener was expressed relative to the maximum response of the 2,3,7,8-TCDD reference curve. Values presented in graphs and tables are presented as mean ± SD.

Reproducibility and repeatability were measured, and the coefficients of variation (%CV) and reproducibility (%VCR) were calculated from log-transformed luminescence data using the following equations (2) and (3):

$$\%CV = 100 * (\%SD/Mean) \qquad (2)$$

$$\%VC_R = 100 * \sqrt{\frac{\sum_{i=1}^{n}(\frac{EC50_{i,1}-EC50_{i,2}}{0,5(EC50_{i,1}+EC50_{i,2})})^2}{2n}} \qquad (3)$$

$EC50x_{i,1}$: log (EC_{50}) i^{th} determination first observation

$EC50x_{i,2}$: log (EC_{50}) i^{th} determination second observation

n : number of determinations

The z-factor was calculated from raw luminescence data using the following equation (4) and is a value (aimed at >0.6 in this study) intending to quantify the suitability of an assay for use in a high-throughput environment (Zhang et al., 1999) :

$$1 - ((3 \times SD_{DMSO} + 3 \times SD_{highest\,[TCDD]})/(Mean_{DMSO} - Mean_{highest[TCDD]})) \quad (4)$$

2.8. Relative potency calculations

The WHO has set four criteria for *in vitro* REP determination (Van den Berg et al., 2006): (i) at least four concentrations of 2,3,7,8-TCDD plus a blank (here DMSO) should be selected, (ii) for both 2,3,7,8-TCDD and the analyzed congener, three of the tested concentrations should elicit a response between the EC_{20} and EC_{80}, (iii) the maximal response (EC_{100}) or plateau should be reached for at least one concentration, (iv) the dose-response curves should be parallel and, (v) the REP value should be based on the EC_{50} of 2,3,7,8-TCDD and the EC_{50} of the congener. When an analyzed congener met those criteria, we determined the half-maximal effect concentration (EC_{50}) and calculated the corresponding REP_{50} value (5):

$$REP_x = EC50_{2,3,7,8-TCDD}/EC50_x \qquad (5)$$

Also, for all analyzed congeners PC5, PC50 and corresponding REP_{PC5}, REP_{PC50} were determined. In this way, we could still determine hypothetic REP values for the congeners that did not elicit a full dose-response curve in the tested range of concentrations and correct for differences observed in maximal ligand efficiency. The PC5 and PC50 values refer to an interpolation of the concentration of the analyzed congener needed to induce activity equal to 5% or 50% of that of 2,3,7,8-TCDD total dose-response (OECD, 2016). All REP values are expressed on a molar basis.

3. Results and discussion

3.1. Development and characterization of a novel DR_{human} CALUX cell line

To develop a human-based AhR reporter gene assay our approach was to first select a human cell line that was sensitive and responsive to the prototypical ligand of the AhR, 2,3,7,8-TCDD and provided easy quantifiable ligand-dependent reporter gene induction after 2,3,7,8-TCDD exposure. To this end, we transiently transfected five human cell lines with the AhR-responsive reporter construct. The U2OS and HEK293 cell lines were selected for their low metabolic capacity and overall low cellular activity that may be relevant for future applications of the bioassay (Peterson et al., 2017; van Vugt-Lussenburg et al., 2018). The C2BBe1 and HT-29 intestinal cell lines were selected for their organ relevance as AhR signalling is thought to play an important role in maintaining intestinal homeostasis (Metidji et al., 2018; Schiering et al., 2017; Veldhoen and Brucklacher-Waldert, 2012). The HepG2 cell line was selected for its organ relevance with respect to dioxins' distribution in the human body, known sensitivity to AhR ligands as well as its popularity for use in high-throughput assays (Ahlborg et al., 1992; Shukla et al., 2010; Westerink et al., 2011). The AhR-responsiveness of the candidate human cell lines were initially screened using 2,3,7,8-TCDD at a concentration of 3E-10M and retained for further testing if 2,3,7,8-TCDD resulted in at least a five-fold induction after 24 hours of exposure.

As shown in Figure 1, C2BBe1 and HepG2 showed significant levels of induction (>5 fold) after 24 hours of exposure to 3E-10M of 2,3,7,8-TCDD, therefore those cell lines were retained for further testing. Transiently transfected C2BBe1 and HepG2 cells were then exposed to a full-dose range of 2,3,7,8-TCDD to determine maximum levels of induction. Although both cell lines showed a similar dose-response for 2,3,7,8-TCDD, the maximal induction of HepG2 upon exposure to 1E-8M of TCDD in the transient experiment was significantly higher (>200-fold) as compared to C2BBe1 (Figure 1). Therefore, we decided to select the HepG2 cell line as the host of choice to develop a stably transfected human AhR-reporter gene assay. Despite the lower level of induction, the C2BBe1 cell line presents a physiologically interesting alternative to the HepG2 liver cells as it would enable us to study AhR transactivation in a gut context where the AhR is thought to play an important physiological role (Metidji et al., 2018; Schiering et al., 2017).

To generate a stable reporter cell line, wild-type HepG2 cells were co-transfected with the AhR-responsive reporter and geneticin resistance plasmids. Following transfection, geneticin-resistant clones were identified by limiting dilution and selected via antibiotic resistance. 2,3,7,8-TCDD-responsive clones were identified, maintained in culture under antibiotic pressure and their responsiveness to 2,3,7,8-TCDD was tested over time at several different passage numbers. Of all candidate clones, clone #2 hereafter named DR_{human} CALUX was found to be stable and highly inducible over 30 passages (Figure 2). To evaluate the stability and reproducibility of the DR_{human} CALUX bioassay several parameters were monitored over an extended time period of continuous culture from

passage 2 to passage 30: the EC_{50} value, the coefficient of variation (%CV) of each EC_{50} determination, the reproducibility variation coefficient (%VCR), coefficient of determination (R^2) and the z-factor (Table 1). The z-factor, as an indicator of data variation and reliability, was aimed to be higher than 0.6 to ensure straightforward and confident quantification also in an automated environment as described by Zhang et al. and applied in the next section (Zhang et al., 1999). The average maximal fold-induction of the DR_{human} CALUX during the testing period was 74-fold and the average EC_{50} was of 3E-10M (n=15), which is in line with previously reported values of 2,3,7,8-TCDD values in human cells (Denison et al., 2004; He et al., 2011). The %CV of each EC_{50} variation was of 1.22% on average and the %VCR was of 0.77%. All R^2 values were higher than 0.98 and all z-factor determinations higher than 0.6 with an average of 0.83.

Overall, the newly developed DR_{human} CALUX cell line appeared to be a highly stable and inducible human-based bioassay with a typically high AhR-sensitivity for 2,3,7,8-TCDD. The reliability of the bioassay enables reproducible measurements that are relevant to analyze AhR-activating properties of individual chemicals and complex mixtures of AhR-interacting chemicals.

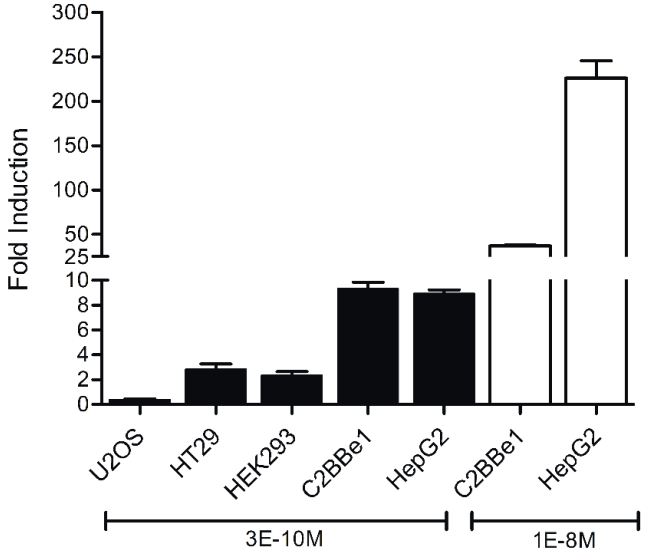

Figure 1. *Responsiveness of human U2OS, HT29, HEK293, C2BBe1 and HepG2 cell lines transiently transfected with an AhR-responsive reporter plasmid to 3E-10M and 10E-9M of 2,3,7,8-TCDD after 24 hours of exposure (mean ± SD).*

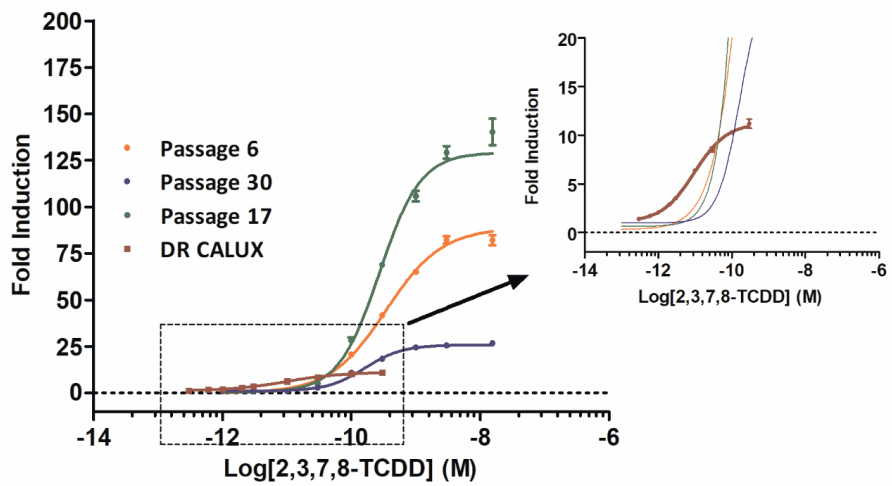

Figure 2. *2,3,7,8-TCDD dose-response curves of the DR_{human} CALUX at three different culture passages as compared to the typical dose-response curve obtained in the rat H4IIe based DR CALUX bioassay (mean ± SD).*

Table 1. *Performance characteristics of the DR_{human} CALUX bioassay in the 96-well plate format and high-throughput screening (HTS) 384-well format (mean ± SD).*

Assay	Average EC_{50} (M)		CV (%)	VC_R (%)	Z-factor	R^2
DR_{human} (n=15)	3.00E-10	± 7E-11	1.22	0.77	0.83 ± 0.19	0.99±0.008
DR_{human} HTS (n=15)	6.00E-10	± 2E-10	2.27	1.0	0.80 0.1	0.99±0.005

3.2. Establishment of an automated high throughput assay format

To facilitate efficient screening, we next established a scale-down from a 96-wells to a 384-wells plate format compatible with an automated high-throughput screening (HTS) method as previously described for other U2OS- and H4IIE-based CALUX cell lines (van Vugt-Lussenburg et al., 2018; 2013). To ensure that results from HTS were comparable with those obtained manually in 96-wells plate format, several parameters were monitored. These parameters were identical to the ones described previously: the EC_{50} variation, the %CV of each EC_{50} determination, R^2 (≥0.98) and the z-factor (≥0.6).

Based on 15 independent high-throughput CALUX analyses (Table 1), the average induction was of 577-fold and the average EC_{50} for 2,3,7,8-TCDD was of 6E-10M (n=15). The %CV of each EC_{50} variation was of 0.95% on average and the %VCR was 1.00%. All

R^2 values were higher than 0.98 and all z-factor determinations above 0.6 with an average of 0.80. These results, compared to those obtained using the bioassay performed in 96-wells plate, show that miniaturization did not have a significant impact on the bioassay performance. Concerning the EC_{50}, a slight difference in sensitivity can be observed in the HTS format as compared to the 96-well plate format, with the miniaturized bioassay being less sensitive than the 96-wells assay. This variation, based on 15 determinations, is considered acceptable since all EC_{50} determinations were in the same order of magnitude as those determined in the 96-well plate format. The main difference, however, between the two set-ups of the bioassay was the maximal induction which was on average higher in the 384-well plate. At present we have no good explanation for this difference but according to the other parameters monitored, it did not affect the performance and reproducibility of the bioassay.

The DR_{human} CALUX bioassay can be used in an HTS-mode for efficient screening of large compound libraries to investigate interactions of dioxins and dioxin-like compounds with the receptor but also interactions of natural, and synthetic health beneficial chemicals with the AhR, which is an area that has gained significant interest over the past decades. Consistent with the literature (Anderson, 1995; Novotna et al., 2011), the DR_{human} CALUX is less sensitive to 2,3,7,8-TCDD than rodent-based bioassays and is, therefore, less suitable for applications requiring high sensitivity, such as monitoring of dioxin toxic equivalents of mixtures in e.g. food matrices. Yet, because the AhR-reporter construct is similar in the DR_{human} CALUX and the rat-H4IIe-based DR CALUX, mixture potency determination by the latter may better reflect the potency of the same mixture for a human individual

Altogether, the DR_{human} CALUX bioassay performed well in an automated and miniaturized format, with high reproducibility between plates, high signal-to-background ratios as well as high z-factor values. We next applied this HTS method to assess the AhR-transactivating potency of individual PBDD/Fs and PCDD/Fs congeners.

3.3. Relative potency (REP) determination of PCDD/Fs and PBDD/Fs

To determine the relative potencies of selected individual PBDD/Fs and PCDD/Fs congeners, we used the automated setup for the DR_{human} and DR CALUX bioassays. All 21 PCDD/F and PBDD/F congeners analyzed in both bioassays showed a dose-dependent increase of luciferase activity confirming the earlier observation that PBDD/Fs *are bona fide* AhR ligands. Although all chlorinated and brominated dioxin congeners induced AhR activity, the tested concentration range did not result in a full dose-response curve for some congeners and we observed some differences in efficacy between congeners. For these reasons, PC values were also calculated, relative to the positive control (2,3,7,8-TCDD). Thus, 5% of 2,3,7,8-TCDD equivalent activity values (PC_5), as well as PC_{50}, were determined when possible (Table 2). Regarding the overall sensitivity to PCDD/Fs, DR_{human} CALUX cells were less sensitive than rat-based DR CALUX cells (Table 2). It has been previously demonstrated that rodents are 10 to 100-fold more sensitive to chlorinated dioxins than humans and in the case of 2,3,7,8-TCDD, we observed a typically

reported 100-fold difference for the EC_{50} between human and rat CALUX cells (He et al., 2011; Long et al., 2003; van den Berg et al., 2013; Wiebel et al., 1996).

Then, we determined the relative potency values based on the PC_5. The PC_5 was chosen instead of the EC_{50} to determine relative potencies as it is the benchmark value where most REPs values could be calculated and because it corresponds to a part of the log-transformed dose-response relationship where the slope is very comparable for all congeners. Indeed, differences in Hill's slope and maximal efficacy between congeners may result in uncertainty when calculating EC_{50}-based REPs values, as suggested by the differences between EC_{50} and PC_{50} values observed in Table 2. The performance of the DR_{human} CALUX bioassay used in this study and the DR CALUX by van Vugt-Lussenburg et al. indicate that the PC_5 can be determined reliably (van Vugt-Lussenburg et al., 2013).

In the case of the DR CALUX (Table 3), the REP_{PC5} values obtained using the automated HTS-mode are similar to those obtained by Behnisch and coworkers in the manually operated 96-well plate assay (Behnisch et al., 2003). The REP_{PC5} values determined in the DR_{human} bioassay shown in Table 4 are in the same range than those obtained by other groups that have been using HepG2-based AhR responsive reporter cell lines to determine PCDD/Fs and PBDD/Fs potencies (Larsson et al., 2015; Olsman et al., 2007). The ranking of REP values found in both bioassays is overall in line with the notion that the REP values decrease with an increase in halogen substitution. We observed that, like PCDD/Fs, PBDD/Fs are potent activators of the human AhR. In HepG2 cells, PBDD/Fs were generally as potent as their chlorinated counterparts, except 1,2,3,4,6,7,8-HpCDD being significantly more potent than its brominated analogue (Table 4). In the rat-based DR CALUX bioassay, PBDD/Fs were also as potent as PCDD/Fs although these seemed to be generally slightly more potent (Table 3).

Deviations between the TEF values used in the chemical-analytical-based determination of TEQ and REPs values of dioxins may lead to a significant underestimation or overestimation of complex mixtures toxicity equivalents. The congener- and species differences we observed then led us to wonder whether the WHO-TEF values, which are based on a REPs database containing almost exclusively rodent-data (Haws et al., 2006), were adapted for TEQ determination of complex mixtures and by extension to human hazard assessment. Particularly in the case of PBDD/Fs for which there are not yet officially assigned toxicity equivalent factors and for which it is recommended to apply TEFs for PCDD/Fs.

Table 2. *PC_5 (5% 2,3,7,8-TCDD activity equivalent), PC_{50} (50% 2,3,7,8-TCDD activity equivalent), EC_{50} and maximal efficacy (%$TCDD_{max}$) values for the PCDD/Fs and PBDD/Fs determined in DR_{human} and DR CALUX cells. Values are expressed in molar (M) and are a mean of at least two independent CALUX analysis performed in triplicates.*

Compound	DR_{human} CALUX				DR CALUX			
	PC_5 (M)	PC_{50} (M)	EC_{50} (M)	%$TCDD_{max}$	PC_5 (M)	PC_{50} (M)	EC_{50} (M)	%$TCDD_{max}$
2,3,7,8-TCDD	2.8E-11	6.0E-10	6.0E-10	100	5.2E-13	6.2E-12	6.2E-12	100
1,2,3,7,8-PeCDD	2.9E-11	3.8E-10	4.4E-10	113	9.5E-13	2.1E-11	1.2E-11	81
1,2,3,4,7,8-HxCDD	2.0E-11	4.2E-10	2.9E-10	100	1.9E-12	3.3E-11	2.6E-11	76
1,2,3,4,6,7,8-HpCDD	7.6E-11	1.1E-09	1.5E-09	115	3.6E-12	1.0E-10	4.9E-11	80
OCDD	nd	nd	nd	4	2.1E-10	6.5E-09	4.0E-09	77
2,3,4,8-TCDF	2.4E-10	3.0E-09	3.6E-09	111	1.4E-12	7.4E-11	2.3E-11	74
2,3,4,7,8-PeCDF	3.0E-11	1.4E-09	5.4E-10	97	1.5E-12	3.8E-11	1.4E-11	79
1,2,3,7,8-PeCDF	4.5E-11	6.7E-10	6.9E-10	103	1.5E-12	9.6E-11	2.7E-11	71
1,2,3,4,7,8-HxCDF	3.5E-11	3.9E-10	4.8E-10	115	1.6E-12	1.2E-10	3.3E-11	69
1,2,3,4,6,7,8-HpCDF	2.6E-09	2.0E-08	2.1E-08	102	9.1E-12	4.9E-10	2.5E-10	81
OCDF	2.4E-08	9.6E-08	nd	62	2.6E-11	5.4E-10	5.2E-10	100
2,3,7,8-TBDD	4.5E-11	5.7E-10	5.5E-10	96	1.1E-12	2.9E-11	2.0E-11	96
1,2,3,7,8-PeBDD	2.2E-11	2.7E-10	2.7E-10	128	1.4E-12	3.3E-11	2.4E-11	90
1,2,3,4,6,7,8-HpBDD	5.3E-09	3.0E-08	2.5E-08	87	3.5E-11	2.3E-09	9.4E-10	104
OBDD	nd	nd	nd	4	2.2E-09	9.8E-08	nd	86
2,3,7,8-TBDF	5.7E-11	8.4E-10	1.0E-09	110	6.4E-13	1.6E-11	7.0E-12	103
2,3,4,7,8-PeBDF	2.4E-11	3.0E-10	4.0E-10	106	1.5E-12	4.9E-11	2.6E-11	71
1,2,3,7,8-PeBDF	1.7E-10	1.8E-09	2.6E-09	129	8.1E-12	1.7E-10	7.1E-11	99
1,2,3,4,7,8-HxBDF	1.4E-10	1.4E-09	1.8E-09	107	1.3E-11	4.9E-10	3.6E-10	107
1,2,3,4,6,7,8-HpBDF	7.3E-09	7.3E-08	2.9E-08	71	9.8E-11	2.0E-09	1.9E-09	88
OBDF	2.4E-08	nd	nd	45	4.7E-10	7.7E-09	7.2E-09	97

nd: not determined

Table 3. *Relative potency values of several PCDD/Fs and PBDD/F congeners calculated based on 5% of 2,3,7,8-TCDD activity (PC_5) in the DR CALUX bioassay and congener-specific ratio $REP_{PCDD/F}/REP_{PBDD/F}$ that illustrate congener-specific differences.*

PCDD/Fs	REP PC_5 (M)	Ratio	REP PC_5 (M)	PBDD/Fs
2,3,7,8-TCDD	1.0	**2**	0.49	**2,3,7,8-TBDD**
1,2,3,7,8-PeCDD	0.54	**1**	0.38	**1,2,3,7,8-PeBDD**
1,2,3,4,6,7,8-HpCDD	0.14	**10**	0.015	**1,2,3,4,6,7,8-HpBDD**
OCDD	0.0025	**11**	0.00023	**OBDD**
2,3,7,8-TCDF	0.38	**0.5**	0.80	**2,3,7,8-TBDF**
2,3,4,7,8-PeCDF	0.34	**1**	0.35	**2,3,4,7,8-PeBDF**
1,2,3,7,8-PeCDF	0.35	**5**	0.064	**1,2,3,7,8-PeBDF**
1,2,3,4,7,8-HxCDF	0.32	**8**	0.040	**1,2,3,4,7,8-HxBDF**
1,2,3,4,6,7,8-HpCDF	0.057	**11**	0.0053	**1,2,3,4,6,7,8-HpBDF**
OCDF	0.020	**18**	0.0011	**OBDF**

nc: not calculated

Table 4. *Relative potency values of several PCDD/Fs and PBDD/F congeners calculated based on 5% of 2,3,7,8-TCDD activity (PC_5) in the DR_{human} CALUX bioassay and congener-specific ratio $REP_{PCDD/F}/REP_{PBDD/F}$ that illustrate congener-specific differences.*

PCDD/Fs	REP PC_5 (M)	Ratio	REP PC_5 (M)	PBDD/Fs
2,3,7,8-TCDD	1.0	**2**	0.63	**2,3,7,8-TBDD**
1,2,3,7,8-PeCDD	0.97	**1**	1.3	**1,2,3,7,8-PeBDD**
1,2,3,4,6,7,8-HpCDD	0.37	**69**	0.0054	**1,2,3,4,6,7,8-HpBDD**
OCDD	nc	-	nc	**OBDD**
2,3,7,8-TCDF	0.12	**0.2**	0.50	**2,3,7,8-TBDF**
2,3,4,7,8-PeCDF	0.96	**1**	1.2	**2,3,4,7,8-PeBDF**
1,2,3,7,8-PeCDF	0.64	**4**	0.17	**1,2,3,7,8-PeBDF**
1,2,3,4,7,8-HxCDF	0.80	**4**	0.20	**1,2,3,4,7,8-HxBDF**
1,2,3,4,6,7,8-HpCDF	0.011	**3**	0.0039	**1,2,3,4,6,7,8-HpBDF**
OCDF	0.0012	**1**	0.0012	**OBDF**

nc: not calculated

3.4. Comparison CALUX REP to WHO-TEF and implications for quantitative hazard assessment

In the previous section, we observed some congener- and species-differences in potencies for PCDD/Fs and PBDD/Fs. Therefore, we determined to what extent TEF values were comparable to human-based potency values and if the default assignment of TEFs to PBDD/Fs based on their chlorinated counterparts, is a valid approach. Particularly considering that the establishment of TEFs was almost exclusively based on rodent-derived REP values (Haws et al., 2006). Table 5 presents, DR_{human} CALUX REP_{EC50} instead of the REP_{PC5} values calculated earlier to facilitate the comparison with WHO-TEF values.

For PCDD/Fs some DR_{human} REP values were in the same order of magnitude as their WHO-TEF value (e.g. less than 10-fold difference) but for congeners 1,2,3,4,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, 1,2,3,7,8-PeCDF and 1,2,3,4,7,8-HxCDF the WHO-TEFs values were 13 to 40-fold lower than DR_{human} REP values as indicated by the ratio calculated in Table 5. Such differences between WHO-TEF and human-based REP values for those congeners were also observed by van Ede et al. and Larsson and co-workers based on measurement of luciferase induction and EROD activity in human peripheral blood lymphocytes from healthy blood donor (Larsson et al., 2015; van Ede et al., 2016, 2014). This indicates that the potency of these congeners towards the human AhR might be significantly underestimated under the actual WHO-TEF scheme. In the case of brominated dioxins and furans, the DR_{human} CALUX REPs were similar to the proposed WHO-TEF values (e.g. ≤10-fold difference observed). According to our results and pending the generation of a larger database of REPs from additional studies, the TEF values assigned to PBDD/Fs seem to be appropriate for human hazard assessment of PBBD/Fs mixtures.

The relatively large deviations we observed between potency values of PCDD/Fs using a human-based DR_{human} CALUX bioassay and WHO-TEFs may pinpoint to the importance of using human cell-derived potency values for a proper evaluation of human health risks associated with exposure to toxic PCDD/Fs and PBDD/Fs.

Table 5. *DR_{human} CALUX REP_{EC50} values and WHO-TEF values for PCDD/Fs and PBDD/Fs and ratio REP_{EC50}/WHO-TEF illustrating the differences between the two values.*

Congener	DR_{human} CALUX REP_{EC50} (M)	WHO TEF (2005,2013)[1]	Ratio DR_{human} REP/ WHO-TEF
TCDD	1.0	1	1
1,2,3,7,8-PeCDD	1.4	1	1
1,2,3,4,7,8-HxCDD	2.0	0.1	20
1,2,3,4,6,7,8-HpCDD	0.40	0.01	40
OCDD	nd	0.0003	nc
2,3,7,8-TCDF	0.17	0.1	2
2,3,4,7,8-PeCDF	1.1	0.3	4
1,2,3,7,8-PeCDF	0.87	0.03	29
1,2,3,4,7,8-HxCDF	1.3	0.1	13
1,2,3,4,6,7,8-HpCDF	0.028	0.01	3
OCDF	nd	0.0003	nc
2,3,7,8-TBDD	1.1	1	1
1,2,3,7,8-PeBDD	2.2	1	2
1,2,3,4,6,7,8-HpBDD	0.024	0.01	2
OBDD	nd	0.0003	nc
2,3,7,8-TBDF	0.60	0.1	6
2,3,4,7,8-PeBDF	1.5	0.3	5
1,2,3,7,8-PeBDF	0.23	0.03	8
1,2,3,4,7,8-HxBDF	0.33	0.1	3
1,2,3,4,6,7,8-HpBDF	0.021	0.01	2
OBDF	nc	0.0003	nc

nd: not determined; nc: not calculated; 1: (Van den Berg et al., 2006) and (van den Berg et al., 2013)

4. Conclusion

In this study, we described and applied a newly developed high-throughput CALUX cell line, the DR_{human} CALUX, which is a HepG2 based variant of the well-known rat-H4IIe-based DR CALUX. The DR_{human} CALUX cell line was found to be a reliable, sensitive, and highly inducible bioassay that is compatible with robot-based automation of the CALUX method. In its 384-well format, the DR_{human} CALUX bioassay can be reliably used for the screening of large compound libraries and toxicological hazards from dioxins and dioxin-like compounds and complex mixtures.

As expected, the human HepG2-based DR_{human} CALUX bioassay was less sensitive to the effects of PCDD/Fs and their brominated analogues than its rodent H4IIe-based DR-CALUX counterpart and we observed congener-specific potency differences between the human and rat-based cell lines that may not be completely explained by species differences in sensitivity. Thus, emphasizing the need for precautions when extrapolating data from animal models with the objective of human hazard assessment. The data obtained also suggest that the potency of certain PCDD/Fs congeners such as 1,2,3,4,7,8-

HxCDD, 1,2,3,4,6,7,8-HpCDD, 1,2,3,7,8-PeCDF, 1,2,3,4,7,8-HxCDF and 1,2,3,4,6,7,8-HpCDF may be underestimated in terms of their toxicological relevance to humans when using the WHO-TEF.

The fact that PBDD/Fs are potent inducers of the human AhR is of concern. Particularly when considering that although their presence is increasingly reported in the environment, they do not fall under any regulation within the European Union. It is urgent to encourage the monitoring of PBDD/Fs and firmly establish the risks related to brominated dioxins exposure. Accordingly, it is crucial to increase the availability of effective (bio)analytical capacities. From that angle, cost-efficient and high-capacity cell-based methods such as DR CALUX® are highly relevant.

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Conflicts of interest

The authors declare no conflict of interest.

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Chapter 3

Detection of high PBDD/Fs levels and dioxin-like activity in toys using a combination of GC-HRMS, rat-based and human-based DR CALUX® reporter gene assays

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Abstract

Brominated dibenzo-p-dioxins and dibenzofurans (PBDD/Fs) are increasingly reported at significant levels in various matrices, including consumer goods that are manufactured from plastics containing certain brominated flame retardants. PBDD/Fs are known ligands for the aryl hydrocarbon receptor (AhR) but are not yet considered in the hazard assessment of dioxin mixtures. The present study aimed to determine if PBDD/Fs levels present in plastic constituents of toys could pose a threat to children's health. PBDD/Fs, unlike their chlorinated counterparts (PCDD/Fs), have not been officially assigned toxic equivalency factors (TEFs) by the WHO therefore, we determined their relative potency towards AhR activation in both human and rodent cell-based DR CALUX® bioassays. This allowed us to compare GC-HRMS PBDD/F congener levels, converted to total toxic equivalency (TEQ) by using the PCDD/F TEFs, to CALUX Bioanalytical Equivalents (BEQ) levels present in contaminated plastic constituents from children's toys. Finally, an estimate was made of the daily ingestion of TEQs from PBDD/Fs-contaminated plastic toys by child mouthing habits. It is observed that the daily ingestion of PBDD/Fs from contaminated plastic toys may significantly contribute to the total dioxin daily intake of young children.

1. Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) are persistent organic pollutants that are highly toxic, particularly regarding developmental toxicity, following exposure at early stages of life (Birnbaum, 1995; Vreugdenhil et al., 2002). Most, if not all toxic effects of dioxins and dioxin-like compounds are mediated via their interaction with the Aryl hydrocarbon Receptor (AhR), which is a key transcription factor involved in numerous biological processes such as xenobiotic metabolism, immune response, and development (Larigot et al., 2018). Because of their detrimental effects on human health, PCDD/Fs are strictly regulated, and low limit values in e.g. food are in force in the EU and other parts of the world.

The standard analytical method for the quantification of dioxins and dioxin-like PCB congeners in various type of samples such as food, house dust and sediments, is capillary Gas Chromatography coupled to High-Resolution Mass Spectrometry (GC-HRMS). To translate quantitative GC-HRMS data into relevant toxicity information, the concentration of each individual toxic PCDD/F congener is multiplied by TEF-based toxic potency and added up to determine the total 2,3,7,8-TCDD related toxic equivalency (TEQ) of a sample (Van den Berg et al., 2006).

Since the turn of the century, innovative cell-based AhR-driven reporter gene assays, such as DR CALUX®, have been developed as an alternative high-capacity screening alternative to GC-HRMS for a wide variety of matrices (Houtman et al., 2002; Murk et al., 1996; Vugt-Lussenburg et al., 2013). The quantification of the total toxic potency of PCDD/Fs, expressed in bioanalytical toxicity equivalence (BEQ), using the DR CALUX bioassay is based on luminescence induction mediated by transactivation of the AhR through the interaction of PCDD/Fs and related compounds. An additional advantage of DR CALUX, and related reporter assays, is that compounds similar to PCDD/Fs in their toxic mode of action, such as brominated dioxins and furans (PBDD/PBDFs) can also be detected and quantified. Combining the bioassay-based BEQ determination with GC-HRMS enables us to either confirm the results chemically or find out which compounds are responsible for the additional bioactivity that may have been detected in a sample, and it this way identify the culprit chemicals, such as PBDD/Fs.

Recently, brominated dioxins (PBDD/Fs) have been generating interest as their presence is increasingly reported in the outdoor and indoor environment (Bjurlid et al., 2018; Choi et al., 2003; Li et al., 2011; Pajurek et al., 2019). PBDD/Fs have been reported to be present at relatively high concentrations in e.g., dust from the indoor environment as described by several studies (Suzuki et al., 2017; Takigami et al., 2009, 2008; Tue et al., 2013). These studies strongly suggest that PBDD/Fs released via dust could represent a possible additional source of exposure for humans to dioxin-like compounds (van den Berg et al., 2013). As an example, PBDD/Fs can be released in an indoor environment from heat-resistant electronics, resulting in elevated PBDD/Fs levels in dust (Takigami et

al., 2008). Brominated dioxins are originally present as impurities in some commercial brominated flame-retardant mixtures, such as decabromodiphenyl ether (DecaBDE) or other PBDE mixtures which are used to flame-retard plastics and electronics (Altarawneh et al., 2019; Buser, 1986; Ren et al., 2017, 2011). Main sources of PBDD/Fs formation are during incomplete thermal degradation of flame-retarded plastics. Unsuitable temperature conditions used during recycling processes may also lead to the formation of PBDD/Fs from brominated flame-retardant precursors (Ebert and Bahadir, 2003; Hamm et al., 2001; Zhan et al., 2019).

A problem arising with the potential presence of PBDD/Fs in plastic is linked to the fact that nowadays, a wide range of consumer products including toys are manufactured from recycled plastics instead of de novo synthesis. For instance, recycled black plastic often indicates plastic originating from e-waste, which is a type of plastics known to contain significant levels of polybrominated diphenyl ethers (PBDEs) and related flame-retardants (Digangi et al., 2017; Drage et al., 2018; Kuang et al., 2018; Samsonek and Puype, 2013; Strakova and Petrlik, 2017). This suggests that consumer products manufactured using black recycled plastic, such as plastic toys, may represent another unsuspected route of exposure to PBDD/Fs via exposure to dust generated by the object or, particularly for young children, via normal mouthing behaviour. Here the focus is on human hazards related to the possible exposure of, young children, to PBDD/PBDF-based contaminants in children's toys made of recycled plastic.

Human hazard assessment of dioxin congeners and mixtures is generally based on extrapolation from rodent models that are widely used because of their sensitivity towards dioxins. Indeed, the remarkable sensitivity of rodent-based models, such as DR CALUX, makes them very suitable for the determination of the total dioxin potency of e.g., mixtures of PCDD/Fs. However, in the objective of human hazard assessment from environmental contaminants, it is essential to also consider human cell-based bioassays, as it is known that differences exist between humans and rodents in terms of sensitivity towards dioxin-related toxicity which may also be reflected in differences in sensitivity of AhR-mediated responses by dioxins and related compounds (Aarts et al., 1995; Brennan et al., 2015; Larsson et al., 2015; Long et al., 2003).

Therefore, we have developed a human variant of the rat-based DR CALUX bioassay in the HepG2 cell line, the DR_{human} CALUX, similar to the one described before by Aarts et al. (1995) to complement rat-based DR CALUX total dioxin-like activity measurement. Providing both sensitive quantification of dioxins and enhanced human-relevance of bioactivity determination.

In this study, we evaluated the presence of PBDD/Fs related dioxin-like toxicity in children's toys using a combination of GC-HRMS and cell-based AhR reporter gene assays (DR and DR_{human} CALUX) to determine toxicity equivalents in plastic toys. First, we determined the responsiveness of PBDD/Fs in both rat DR CALUX and its human variant in HepG2 cells, DR_{human} CALUX, we then quantified PBDD/Fs levels by GC-HRMS and

calculated related TEQ levels by using the TEFs recommended by the WHO. Following quantification and TEQ determination by GC-HRMS, we determined bioanalytical equivalents in the toy samples using DR and DR_{human} CALUX. Finally, we estimated the potential contribution of PBDD/Fs observed in the plastic toys to the total daily dioxin intake in young children.

2. Material and methods

2.1. Chemicals

For the analysis of individual congeners using the bioassays, 2,3,7,8-TBDD, 2,3,7,8-TBDF, 2,3,4,7,8-PeBDF, 1,2,3,4,6,7,8-HpBDF and OBDF were purchased as analytical standards from Wellington Laboratories (Guelph, Canada). Congeners 2,3,7,8-TCDD and 1,2,3,4,7,8-HxBDF were purchased as analytical standards from Cambridge Isotope Laboratories (Andover, MA USA). Dimethyl Sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium) and the n-Hexane was purchased from Biosolve (Valkenswaard, The Netherlands). All $^{13}C_{12}$-labeled PBDD/Fs standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Samples and sample preparation

The toys subset selected for this study originated from a larger campaign of plastic consumer product sampling that took place between 2016 and 2018. All 6 selected toys contained a total PBDE content (>500ppm) and Deca-BDE content (>250 ppm) in their black component (Strakova et al., 2018). All toys were made in China but purchased in different countries. Sample 1 was a puzzle cube from Argentina, Sample 2 was a hair clip from the Czech Republic, Sample 3 a key fob puzzle cube, Sample 4 and 5 were puzzle cubes from India and Nigeria, respectively. Sample 6 was a toy guitar from Portugal. The exact composition of the toys is not known. However, considering the potential e-waste origin of the plastic used to manufacture the toys, it is very likely that acrylonitrile butadiene styrene (ABS) is the major component of the plastic.

Before the extraction, we separated black plastic parts from the rest of the toy, since it is this type of plastic that is suspected to contain the polybrominated diphenyl ether (PBDEs)-type flame retardants and therefore are suspect of contamination with PBDD/Fs (Digangi and Strakova, 2016; Turner, 2018). After separation, the black plastic was cut in small pieces and between 5 g and 30 g of black plastic material were transferred into a glass bottle (Schott, Mainz, Germany) and 30 mL to 60 mL of n-hexane was added, covering the plastic material completely. Each sample was then further broken down in small particles and homogenized with a homogenization apparatus (IKA Ultra-turrax, Staufen, Germany). Following 24 hours of extraction in n-hexane, the organic solvent fraction containing the compounds of interest was collected and the sample extracted two times more with newly added n-hexane. Finally, all n-hexane extractions were pooled and evaporated to approximatively 1 mL under a N_2 flow using a solvent evaporator (Dionex, Sunnyvale, CA USA). The remaining 1 mL solvent was cleaned-up twice by sulphuric acid silica gel columns. Finally, part of the cleaned extract dissolved in n-hexane

was used for GC-HRMS analysis and the other part was evaporated and transferred to DMSO for CALUX analysis.

2.3. GC-HRMS analysis for PBDD/Fs

The GC-HRMS analysis for PBDD/Fs was performed by an ISO 17025:2005 accredited GC-HRMS laboratory (MAS GmbH, Münster, Germany) (Hamm et al., 2001; Imai et al., 2003). All PBDD/F analyses by MAS were performed by using the accredited test method MAS_PA002:2013-10 "Determination of the mass concentration of PCDD/Fs, PBDD/Fs and dioxin-like PCBs in solid matter samples". For 1,2,3,6,7,8-HxBDF, 2,3,4,6,7,8-HxBDF and 1,2,3,4,7,8,9-HpBDF congeners neither native nor isotope-labelled standards were commercially available, thus the assignment of the peak signals of these congeners was accomplished by relative retention time comparison with corresponding chlorinated dibenzofurans (Donnelly et al., 1991). As suggested by the WHO, toxic equivalency factors (TEF) for PCDD/Fs were used as a surrogate for calculation of WHO-TEQ levels of PBDD/Fs in samples (van den Berg et al., 2013).

Aliquots of sample extracts sent by BDS to the MAS laboratory were fortified with $^{13}C_{12}$-labeled PBDD/Fs standards and further purified by several liquid chromatography clean-up steps. Prior to GC-HRMS analysis, additional $^{13}C_{12}$-labeled PBDD/Fs standards were added to the PBDD/F fractions as recovery standards (Supplementary data 1). For PBDD/Fs analysis, a capillary gas-chromatograph (GC) (Thermo Scientific GC-Ultra), coupled with a high-resolution mass spectrometer (HRMS) (Thermo Scientific MAT 95XP HRMS) was used. The GC was equipped with a Programmable Temperature Vaporizer injection port and a 30 m DB-5MS capillary column (Agilent J&W GC column, 0.25 mm inner diameter, and 0.1 µm film thickness). The MS was operated in Selected Ion Monitoring (SIM) mode to monitor selected masses of the molecular ion cluster. Additionally, masses of the molecular ions of PBDEs were monitored to check for potential co-elution of PBDEs with PBDFs, which can lead to false positives results. Native PBDD/F congeners were quantified via the internal $^{13}C_{12}$-labeled PBDD/F standards. Due to the large concentration gradients of the PBDFs in the samples, the higher brominated dibenzofurans partly had to be determined by separate analyses of diluted extract aliquots. Chromatograms of such determinations are given, along with other congener's chromatograms, in Supplementary data 2 for Sample 4 as an example.

2.4. Cell lines and culturing

The DR CALUX cell line (Murk et al., 1996) and the DR_{human} CALUX cell line were used for the analysis of individual congeners and samples. The DR CALUX cell line represents an H4IIe-based (rat) cell line stably transfected with the pGudLuc1.1 AhR-controlled luciferase reporter plasmid. The DR_{human} CALUX cell line is a human variant of the DR CALUX and was created in a human HepG2 cell line after stably transfection with the same pGudLuc1.1 AhR reporter construct. DR CALUX and DR_{human} CALUX cells were cultured in α-MEM (Gibco) medium supplemented with 10% fetal calf serum (FCS) and DMEM:F-12 (Gibco) medium that were supplemented with 7.5% FCS, 10% non-essential amino

acids (NEAA), respectively, and streptomycin (10 µg/mL) plus penicillin (10 U/mL) antibiotics. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

2.5. CALUX analyses of pure compounds and extracts from toys and consumer products

In the case of pure compound analysis, the automated CALUX bioassays were carried out as described earlier (van der Burg et al., 2013). Briefly, DR and DR_{human} CALUX reporter cells were manually distributed over a white 384-wells plate and the plates placed in an incubator. After 24 hours, reporter cells (70-95% confluence) were retrieved and the exposure medium was prepared. Using a liquid handling robot (Hamilton Starlet, Hamilton, USA), the dilution series in 0.5 log unit increments of each test compound (dissolved in DMSO) were prepared in the assay medium. Then, the liquid handling robot used the dilutions series to expose the cells. All compounds were tested in triplicate and the same exposure medium was used for both DR and DR_{human} CALUX. Following 24 hours of incubation, the cells were lysed using Triton lysis buffer. Ultimately, the luciferase activity was measured using a luminometer (Berthold, Bad Wildbad, Germany) after the addition of a luciferin-containing solution.

In the case of the toy samples, the DR CALUX analysis was performed in the same way but in a 96-wells format. In this set-up, the preparation of the compound dilution series, as well as the exposure of the DR CALUX cells, were performed manually by an operator instead of a robot.

2.6. Data analysis and quality control of CALUX analysis

2.6.1. Analysis of individual congeners

Each congener was analyzed in triplicate in two independent CALUX analysis performed using the method described above. On each plate, a complete 13-points 2,3,7,8-TCDD reference dilution series was analyzed in two duplicates (n=4) and the rest of the plate was used to analyses a 13-points full-concentration range of a maximum of 6 compounds.

For analysis of results, the software GraphPad Prism was used for dose-response modelling employing a four parameters nonlinear regression model (1):

$$(Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10\text{^}((\text{LogEC50} - X) \times \text{HillSlope}) \quad (1)$$

Using raw luminescence data, the maximum response of the reference compound 2,3,7,8-TCDD was set at 100% and the response of individual congener was expressed relative to the maximum response of the 2,3,7,8-TCDD reference curve. When reached, the half-maximal effective concentration (EC_{50}) of the tested compounds was determined from the dose-response curve.

2.6.2. Determination of relative potency values

Relative potency values were determined from the fitted dose-response curve of the analyzed compounds. Relative potency values were calculated by dividing the $EC_{10\%\text{-TCDD}}$

or EC_{50} of 2,3,7,8-TCDD by the $EC_{10\%}$ or EC_{50} of the PBDD/F congener. The $EC_{10\%}$ value refers to an interpolation of the concentration of the analyzed congener needed to induce activity equal to 10% of that of 2,3,7,8-TCDD total dose-response. Relative potency values were determined for both DR and DR_{human} CALUX and are expressed on a molar basis.

2.6.3. Sample extracts analysis and BEQ determination

Each sample was analyzed in triplicate twice in two independent CALUX analyses. On each 96-well plate, a complete 10-points 2,3,7,8-TCDD concentration range was analyzed in triplicates and the rest of the plate, excluding the outer wells, was used to analyses a dilution range of the sample. For both DR and DR_{human} CALUX bioassays, several parameters were checked to verify the validity of the results: (i) R^2 of standard curve > 0.98, (ii) z-factor of standard curve > 0.6, (iii) 2,3,7,8-TCDD EC_{50} between assay-specific predetermined limit values and (iv) SD of analyzed triplicate < 15%. Then, relative light units from the samples were interpolated from the 2,3,7,8-TCDD standard dose-response curve of the plate and the CALUX BEQ content quantified between the limit of quantification and the EC_{50} of 2,3,7,8-TCDD and, as close as possible to the EC_{10} (Besselink et al., 2004).

3. Results & Discussion

3.1. Determination of relative potencies of individual PBDD/F and PCDD/F congeners in rat- and human-cell based CALUX bioassays

Unlike PCDD/Fs, PBDD/Fs are not assigned official WHO-TEF values. However, to allow the determination of PBDD/Fs-related toxicity equivalents, a WHO expert panel recently recommended using the TEF value normally assigned to the corresponding chlorinated congener for its brominated equivalent (van den Berg et al., 2013). Before determining the dioxin-like activity in the toys using CALUX bioassays, we decided to first investigate if PBDD/Fs TEF values were comparable to rat- and human-cell based CALUX Relative Potency (REP) values and consequently if a comparison between analytical and bioassay TEQ would be meaningful. To assess the potential AhR-dependent luciferase expression by PBDD/Fs, we analyzed a selection of PBDD/Fs congeners using both the well-established DR CALUX and its human variant, the DR_{human} CALUX bioassay. We selected six PBDD/Fs congeners that were either representative of congener's prevalence in the toys (mainly PBDFs, as seen later in Table 2) (2,3,7,8-PeBDF, 1,2,3,4,7,9-HxBDF, 1,2,3,4,6,7,8-HpBDF and OBDF) or structurally similar to 2,3,7,8-TCDD (2,3,7,8,-TBDD and 2,3,7,8-TBDF).

All compounds were active in a dose-dependent manner in both DR and DR_{human} CALUX bioassay. Therefore, it was possible to determine LOEC and $EC_{10\%}$ values for each compound (Table 1). In the case of compound displaying a full dose-response curve, we also determined the EC_{50} half-maximal concentration. With the DR CALUX bioassay, Suzuki et al. (Suzuki et al., 2017) and Behnisch et al. (Behnisch et al., 2003) obtained similar relative potency values for PBDD/Fs and a clear trend of potency decrease with bromine substitution increase can be seen. Contrastingly, this trend for potencies is less

evident in the case of the DR_{human} CALUX where REP_{EC50} values, except for 1,2,3,4,5,7,8-HpBDF and OBDF, are relatively close to each other. Differences in potencies and sensitivity between DR CALUX and DR_{human} CALUX can clearly be seen in Figure 1, indicating some species differences regarding PBDD/Fs bioactivity. The rat cell-based bioassay (DR CALUX) appeared to be more sensitive to PBDD/Fs than its human variant and we observed an overall 100-fold difference based on the EC_{50} value of 2,3,7,8-TCDD and between 10 to 100-fold difference for the analyzed PBDD/Fs between the two cell lines. This observation is in line with the notion, for chlorinated dioxins, that humans are, compare to rodents, relatively insensitive to dioxins (He et al., 2011; Long et al., 2003; van den Berg et al., 2013; Wiebel et al., 1996)

We then compared the CALUX $REP_{EC10\%}$ and REP_{EC50} values determined in both bioassays to the TEF values for PBDD/Fs hazard assessment which are, for the recall, derived from the TEF values for chlorinated dioxins. The TEF values for this set of PBDD/Fs congener appear to be roughly in the same order of magnitude than the $REP_{EC10\%}$ and REP_{EC50} values determined with the DR and DR_{human} CALUX. This indicates that PBDD/Fs-related GC-HRMS estimated TEQ and DR/DR_{human} CALUX BEQ could potentially be compared. This, however, should be verified with a larger set of PBDD/Fs reference compounds.

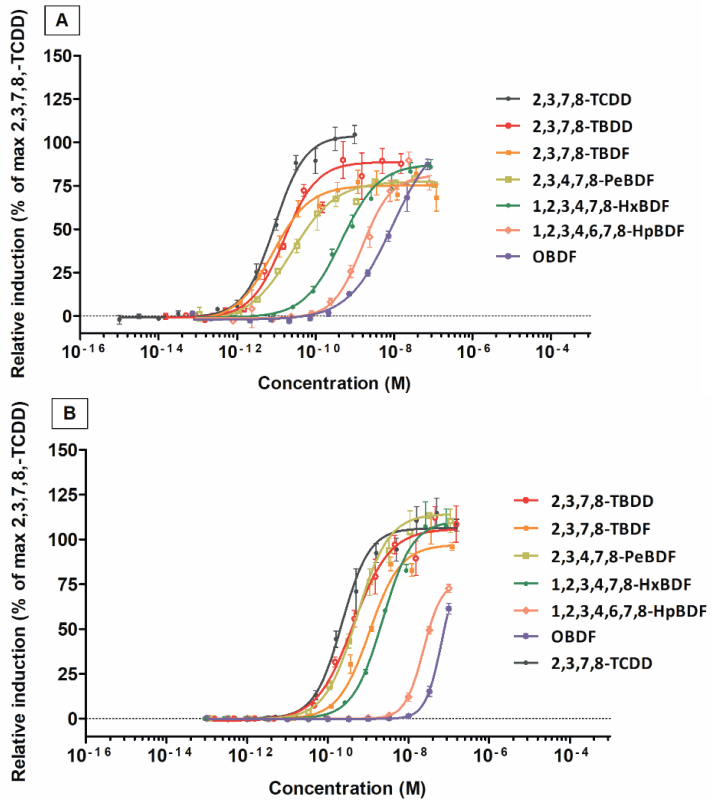

Figure 1. *Typical dose-response curve for several chlorinated and brominated dibenzo-p-dioxins and dibenzofuran in DR CALUX (A) and DR_{human} CALUX (B) (n ≥ 2). Values represent the mean ± SD.*

Table 1. *Lowest effective concentration (LOEC), $EC_{10\%}$, EC_{50} and Relative Potency (REP) values of several PBDD/Fs determined in the DR and DR_{human} CALUX bioassays.*

Compound	DR CALUX				DR_{human} CALUX				WHO TEF (2005)[1]
	LOEC (M)	$EC_{10\%}$ (M)	REP $EC_{10\%}$	EC_{50} (M)	REP EC_{50}	LOEC (M)	$EC_{10\%}$ (M)	REP $EC_{10\%}$	EC_{50} (M)	REP EC_{50}	
2,3,7,8-TCDD	6.0E-13	5.7E-13	**1**	5.8E-12	**1**	1.5E-11	1.0E-10	**1**	4.8E-10	**1**	1
2,3,7,8-TBDD	2.6E-12	1.7E-12	**0.23**	2.5E-11	**0.23**	3.9E-12	6.0E-11	**0.17**	4.5E-10	**0.11**	1
2,3,7,8-TBDF	5.6E-13	8.9E-13	**1.1**	6.7E-12	**0.86**	6.1E-12	9.0E-11	**1.1**	1.2E-09	**0.37**	0.1
2,3,4,7,8-PeBDF	2.0E-12	7.0E-13	**0.3**	3.3E-11	**0.18**	7.1E-12	5.9E-11	**1.69**	4.9E-10	**0.98**	0.3
1,2,3,4,7,8-HxBDF	1.0E-11	2.1E-11	**0.058**	7.4E-10	**0.0078**	2.8E-11	4.1E-10	**0.24**	1.6E-09	**0.3**	0.1
1,2,3,4,6,7,8-HpBDF	2.2E-10	3.0E-10	**0.0028**	2.0E-09	**0.0029**	1.9E-09	1.3E-08	**0.0074**	2.8E-08	**0.017**	0.01
OBDF	6.7E-10	3.2E-10	**0.0009**	2.8E-09	**0.0021**	9.2E-09	4.8E-08	**0.0021**	nd[1]	**nd[1]**	0.0003

nd: EC_{50} could not be determined based on the fit of the curve (absence of plateau in the dose-response); 1: the TEF values presented are recommended TEF values for PBDD/Fs and derived from TEF values of the corresponding chlorinated congener (van den Berg et al., 2013).

3.2. Determination of PBDD/Fs levels (by GC-HRMS), and total dioxin-like activities in toy samples using DRs CALUX bioassays

As described earlier, black plastic parts from toys sampled in several countries selected by their PBDEs content (>500 ppm) and DecaBDE content (>250ppm) (Strakova et al., 2018) were extracted and analyzed for PBDD/Fs. The samples were not analyzed for PCDD/Fs content. PBDD/Fs were found in all samples and high total levels of 17 PBDD/Fs congeners were measured, ranging from 5594 pg/g to 385856 pg/g (Table 2). The distribution pattern was comparable between the samples and PBDFs were the most abundant congeners, accounting for 98.8% to 100% of the total profile. Particularly octa-, hepta-, and hexa-BDFs that were detected in every sample (Figure 2, A). This predominance of PBDFs in plastics is in line with previous findings. Indeed, the PBDD/Fs pattern found in the toys does resemble the pattern of PBDD/Fs impurities found in some DecaBDE mixtures (Ren et al., 2011) used as a flame-retardant in plastics. This suggests that the recycling of DecaBDE containing plastics potentially also allows the recycling of significant amounts of PBDD/Fs into new products (Strakova et al., 2018). Additionally, the formation of PBDFs from DecaBDE also requires low amounts of energy and could, therefore, occur during plastic recycling process (Ebert and Bahadir, 2003; Hamm et al., 2001) leading to increasing PBDD/Fs levels in plastics.

From the PBDD/Fs concentrations determined by GC-HRMS analysis, we estimated the toxicity equivalent in the samples. The corresponding PBDD/Fs-based TEQ concentrations were estimated and ranged from 38 pg TEQ/g to 3384 pg TEQ/g (Table 3). As was the case for PBDD/Fs congener levels, the distribution pattern of PBDD/Fs relative to their contribution to the total ΣPBDD/Fs TEQ levels was similar between the analyzed samples and PBDF were accounting for 96.7 to 100% of the TEQ. Clearly, 1,2,3,4,6,7,8-HpBDF was found to significantly contribute to the overall TEQ of the mixture (Figure 2, B). After determining PBDD/F levels with GC-HRMS and calculating of PBDD/Fs-related TEQ levels, we determined the total dioxin-like bioactivity in the samples using both DR CALUX and DR_{human} CALUX bioassays. CALUX activities were found in all samples tested and, the total dioxin-like activity ranged from 230 to 1520 pg 2,3,7,8-TCDD BEQ/g for the DR CALUX and from 640 to 2550 pg 2,3,7,8-TCDD BEQ/g for the DR_{human} CALUX (Table 3). Some differences are observed when comparing GC-HRMS-based PBDD/Fs TEQs and total bioanalytical equivalents.

To investigate these differences, we calculated theoretical BEQ (BEQ_T) values for the samples based on previously determined congener-specific $REP_{EC10\%}$ values, TEF values for non-analyzed congeners and GC-HRMS concentrations of PBDD/Fs (Table 2). Comparison between GC-HRMS-based TEQs and DR CALUX BEQ_T values shows comparable values and lower $BEQs_T$ values can potentially be explained by CALUX $REP_{EC10\%}$-TCDD being generally lower than TEF values. In the case of DR_{human} CALUX, $BEQs_T$ are higher than TEQs, which can also be explained by differences between REP values and WHO-TEF as DR_{human} are overall higher than WHO-TEF values. Clearly, $BEQs_T$

values show that PBDD/Fs-related BEQ explained most of the total CALUX activities observed.

Differences between TEQs/BEQ_T and the total BEQ values may be explained by the presence of other, unknown compounds in the mixtures, acting either as agonists or antagonists of the AhR. In our case, it is expected that PBDEs present in the samples can partially explain the difference between TEQs/BEQ_T and BEQ. Unlike dioxins, PBDEs are not exclusively agonists of the AhR and some of them are capable to antagonize the AhR (Behnisch et al., 2003; Hamers et al., 2006; Peters et al., 2004). Therefore, since PBDEs have different potencies in human and rodent cell lines, their effect on the total mixture activity is expected to result in different BEQs determination in rodent versus human-based CALUX bioassays.

The suitability of the DR CALUX to accurately determine toxicity equivalence in complex mixtures of chlorinated dioxins and dioxin-like compounds has been demonstrated before (Besselink et al., 2004; Croes et al., 2013; Gizzi et al., 2005; Vugt-Lussenburg et al., 2013) however, it is equally important to consider the toxicological relevance of the total bioactivity determination. A bioassay-determined activity in a sample is the result of complex toxicological interactions (additive, non-additive, antagonism, synergism…) which cannot easily be predicted using an analytical-chemical method. Because these interactions can differ between species, the use of the DR_{human} CALUX bioassay is relevant in terms of human hazard assessment based on the effect of complex mixtures on the human AhR. From that angle, the DR_{human} CALUX bioassay can be seen as a complementary tool to sensitive DR CALUX activity determination for increased human relevance.

Table 2. *Concentrations of PBDD/F (pg/g) congeners as measured by GC-HRMS in plastic materials from toys; total sample concentration (pg/g) and WHO-2005 based TEQ values measured in plastic material from toys using GC-HRMS.*

Congener	WHO TEF (2005)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
2,3,7,8-TBDD[2]	1	3.69	< 4[1]	< 8[1]	2.23	< 2[1]	< 5[1]
1,2,3,7,8-PeBDD[2]	1	15.9	< 16[1]	< 32[1]	9.18	< 8[1]	< 20[1]
1,2,3,4,7,8/1,2,3,6,7,8-HxBDD[2,4]	0.1	24.2	< 32[1]	< 64[1]	14.4	< 16[1]	54.4
1,2,3,7,8,9-HxBDD[2]	0.1	< 16[1]	< 32[1]	< 64[1]	10.7	< 16[1]	< 40[1]
1,2,3,4,6,7,8-HpBDD	0.01	359	< 200[1]	1861	226	< 100[1]	612
OBDD	0.0003	42.8	< 4[1]	230	29.7	5.84	227
2,3,7,8-TBDF[2]	0.1	65.5	< 16[1]	451	66.1	< 8[1]	223
1,2,3,7,8-PeBDF[2]	0.03	95.4	< 16[1]	856	69.9	15.4	596
2,3,4,7,8-PeBDF[2]	0.3	780	98	6759	1188	101	2187
1,2,3,7,8,9-HxBDF[2]	0.1	< 16[1]	< 32[1]	445	< 8[1]	< 16[1]	93.3
2,3,4,6,7,8-HxBDF[2,3]	0.1	358	32.5	4855	399	59.4	1714
2,3,4,6,7,8-HxBDF[2,3]	0.1	358	32.5	4855	399	59.4	1714
1,2,3,4,6,7,8-HpBDF	0.01	51478	2751	226399	47937	2569	50476
1,2,3,4,7,8,9-HpBDF[3]	0.01	296	< 80[1]	464	156	< 40[1]	140
OBDF	0.0003	117070	2712	142586	37426	3943	41964
TOTAL (pg/g)		***170753***	***5594***	***385856***	***87716***	***6694***	***98745***
TOTAL (pg TEQ/g)[5]		***725***	***41***	***3821***	***693***	***48***	***1137***

1: not detected above specified the limit of quantification; 2:data for these congeners represent maximum values since co-elution with other congeners of the same homologue group cannot be excluded; 3: due to uncertainty in the assignment of these congeners, results have to be considered as tentative for these congeners; 4: co-eluting congeners; 5: as no TEF values have been officially established for PBDD/Fs, the TEQ values presented were calculated by using the WHO-2005 TEFs from their chlorinated analogues PCDD/Fs.

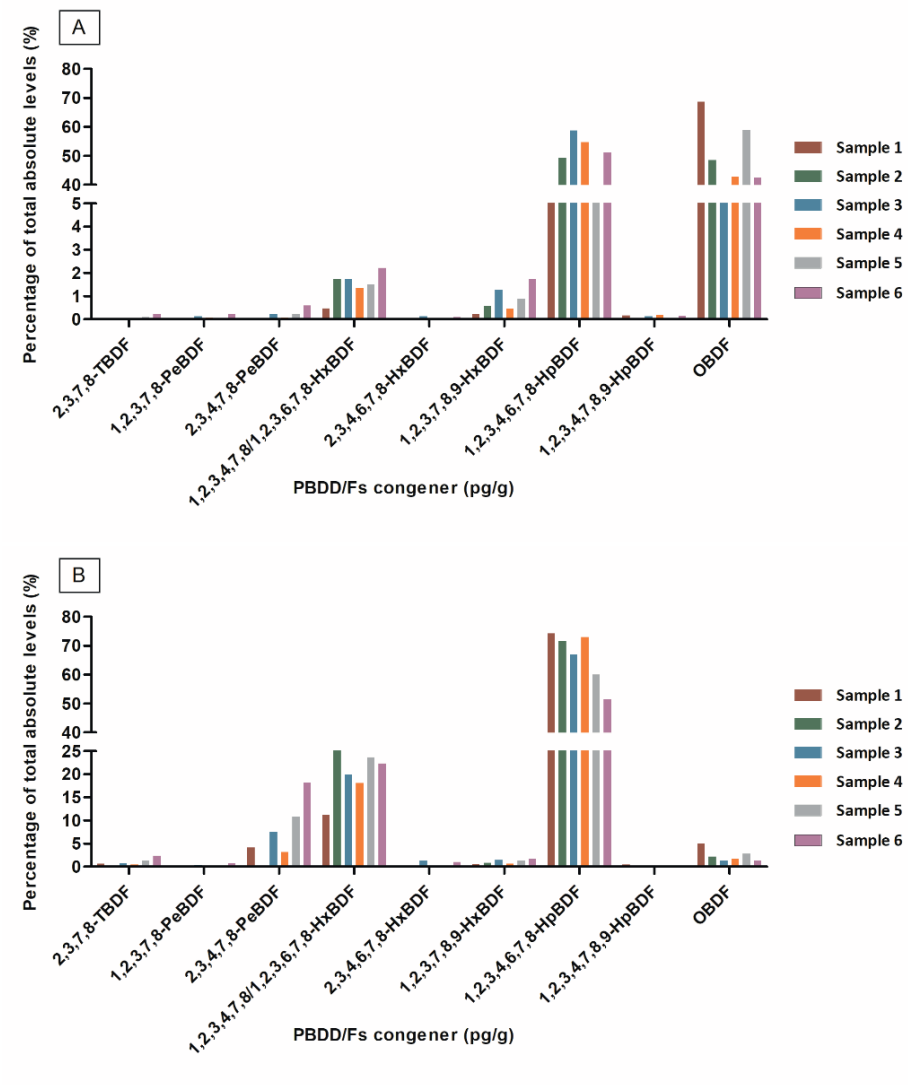

Figure 2. *PBDFs congeners pattern in six toys measured by HR-GCMS presented in percentage of contribution to the total concentration of PBDFs (pg/g) (A) and percentage of contribution to the total TEQ (B).*

Table 3. *Total equivalent activity (TEQ) values measured in analyzed toys and consumer goods using GC-HRMS (pg 2,3,7,8-TCDD TEQ/g), as well as DR CALUX and DR_{human} CALUX (pg 2,3,7,8-TCDD BEQ/g).*

Sample ID	HR-GC/MS TEQ[1] (pg TCDD TEQ/g)	DR CALUX BEQ (pg TCDD BEQ/g)	Theroretical DR CALUX BEQ[2] (pg TCDD BEQ/g)	DR_{human} CALUX BEQ (pg TCDD BEQ/g)	Theoretical DR_{human} CALUX BEQ[2] (pg TCDD BEQ/g)
Sample 1	725	1370	432	1230	1089
Sample 2	41	230	19	680	53
Sample 3	3821	1520	2222	2550	5855
Sample 4	693	550	348	890	930
Sample 5	48	370	34	650	90
Sample 6	1137	370	933	640	2442

1: as no TEF values have been officially established for PBDD/Fs, the TEQ values presented were calculated by using the WHO-2005 TEF values for the chlorinated analogues PCDD/Fs; 2: Theoretical CALUX BEQ values were determined based on the $REP_{EC10\%\text{-}TCDD}$ determined in this study and their resulting TEF values for PBDD/Fs.

3.3. Hazard assessment of plastic toys contaminant uptake by children through mouthing behaviour

It is worthy to note that a value of 1000 pg 2,3,7,8-TCDD TEQ/g as can be found in plastic toys, is equivalent to the proposed hazardous waste limit for chlorinated dioxins in the Stockholm Convention for Persistent Organic Pollutants (Petrlik, 2019). Considering this limit, toys 1, 3 and 6 were found to be as contaminated as hazardous waste. All other samples were also highly contaminated. Such high levels of PBDD/Fs contamination in consumer products indicate that they were most likely manufactured using flame retardant containing recycled plastics. The existence of this amount and type of contamination suggests a potential additional route of exposure of e.g., children to PBDD/Fs and other hazardous chemicals, such as PBDEs, which apparently is associated with the current way of recycling of plastics, and is very relevant with respect to the ambition of creating a global circular economy.

To estimate the potential for possible harm from plastic toys contaminated with relatively high PBDD/F levels, we have executed a first approximation of a hazard-assessment, bearing in mind the mouthing behaviour as a possible route of exposure for children. It is known that for children under the age of 3, mouthing behaviour plays an important role regarding the contact of children to toy materials (van Engelen et al., 2007). The ingestion of plastic that is scraped off toys can then be an important route of exposure of young children to contaminants in plastics, including dioxins and dioxin-like compounds. In 2016, the European Scientific Committee on Health and Environmental Risks (ESCHER) reconfirmed the validity of the amounts ingested by children from toys by mouthing behaviour as defined in a report from the Dutch National Institute for Public Health and the Environment (RIVM) (van Engelen et al., 2009). In the ESCHER report (Ms Scientific Committee SCHER et al., 2016), the daily ingestion of scraped-off plastic toy material is estimated at 8 mg/day; 100 mg/day for dry, brittle, powder-like or pliable toy material, and 400 mg/day for liquid or sticky toy material. In this first approximation of hazard assessment, we used the lower value of 8 mg/day ingestion of scrapped-off plastic toy material. Because of the very limited data available on PBDD/Fs bioavailability, we assumed as a worst-case scenario that 100% of the PBDD/Fs would be bioavailable from the ingested plastic and absorbed over the intestinal tract. This is in line with the original RIVM report which states that in the scenario of direct ingestion and mouthing, 100% of the elements would migrate out of the plastic over the intestinal tract (van Engelen et al., 2009).

By using the values of previously determined GC-HRMS PBDD/Fs-based TEQs and DR/DR_{human} CALUX bioassay BEQs in toys and the average body weight values for children of 6, 12, 24 and 36 months, we estimated the possible daily intake of PBDD/Fs related to the ingestion of 8 mg scrapped contaminated plastic toy material expressed as pg TEQ/kg body weight/day (Figure 3, Supplementary Table 3). Considering the recommended tolerable daily intake (TDI) for dioxins of 0.28 pg TEQ/kg body weight/day, our estimated contribution of the ingestion of contaminated black plastic to

children's dioxin body burden is significant and often higher than the recommended TDI for PCDD/Fs and dioxin-like PCBs which we can use for comparison with exposure to PBDD/Fs. In the case of GC-HRMS PBDD/Fs-based TEQ and DR CALUX BEQ, two toys (Sample 2 and 5) were below the TDI at all ages (Figure 3A and 3B). In the case of the DR_{human} CALUX BEQ, bioassay representative of the activity of the mixture on the human AhR, none of the toys was below the TDI at all age (Figure 3C). As an example, the ingestion of 8 mg of black plastic scrapped off Sample 3 by a 12-month-old toddler could potentially result in the ingestion of 2.27 pg BEQ/kg body weight/day based on DR_{human} CALUX BEQ activity. This represents an intake of 2,3,7,8-TCDD equivalents which are 9 times higher than the recommended TDI for dioxins of 0.28 pg TEQ/kg body weight/day (Knutsen et al., 2018). It is important to emphasize that this estimation only focuses on PBDD/Fs and does not include the dietary intake of chlorinated dioxins, therefore our finding supports the argument that PBDD/Fs can "contribute significantly to the total amount of TEQ" (van den Berg et al., 2013), and should, therefore, in our opinion be included into overall dioxin-like toxicity estimations for a better hazard assessment of the total TEQ by chromatography-based methods.

However, this hazard assessment study has potential limitations. As we could not pulverize the plastic into a fine powder, it is expected that PBDD/Fs extraction is incomplete. On the other hand, the dimension of the particles obtained was representative of what can be scrapped and swallowed by a child. Additionally, the fact that we only focused on the black plastic parts of the toys is a limitation, however, it should be noted that the toys analyzed were anyway essentially made of black plastic and that these parts were visible and accessible for a child for hand-to-mouth contact. Another potential limitation is that the type of toys analyzed may not be intended for children over 3 years old. Nevertheless, children under 3 years would likely have access to them as their caretakers would probably not keep such toys out of reach from a child displaying mouthing behaviour. In other cases, behavioural studies show that children mouth on a broad range of items not intended to be mouthed, and thus they could have access to other potentially contaminated objects (Juberg et al., 2001; Smith and Norris, 2003). Finally, congener-specific bioavailability was not considered and, based on PCDD/Fs studies, it is anticipated that the bioavailability of higher brominated congeners will be lower than for lower substitution congeners. Clearly, more studies investigating the bioavailability of PBDD/Fs from plastics are needed.

Overall, our findings suggest that the use of PBDD/Fs contaminated plastic could potentially represent a significant additional source and route of exposure for young children to hazardous compounds. Furthermore, since nowadays it is stimulated globally to recycle plastic for renewed product formation and use, the potential contamination of plastics by brominated flame retardants and associated PBDD/Fs needs to be reconsidered. A solution could be the monitoring of PBDD/Fs levels in recycled plastics and removal of such contaminant-containing parts prior to their use for the manufacturing of new consumer products.

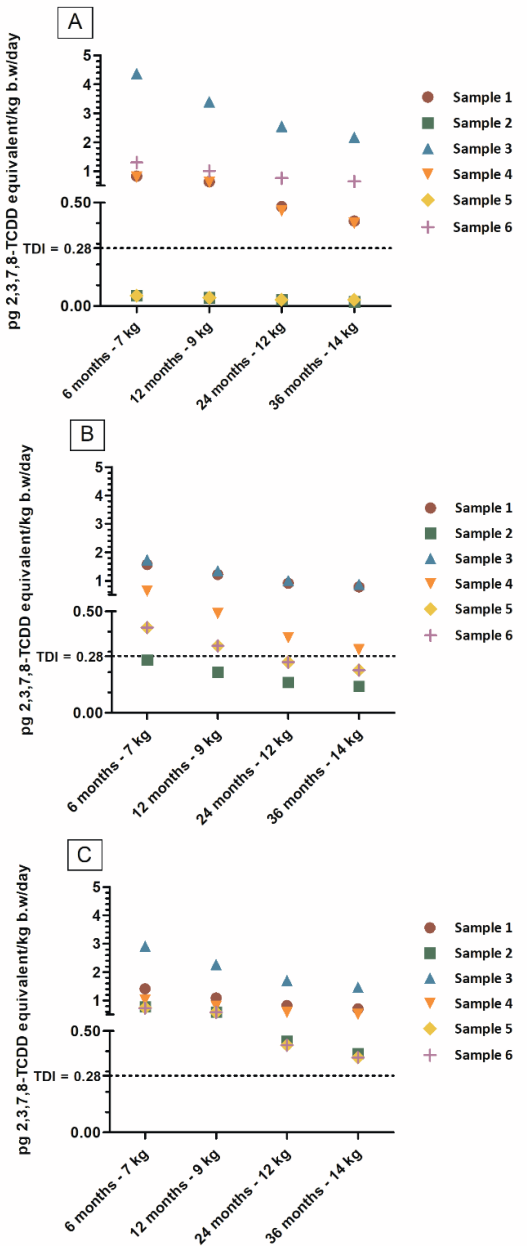

Figure 3. *Estimation of the additional daily intake in young children of PBDD/Fs in plastic toys from mouthing behaviour. Estimation of the pg TEQ/kg body weight/day is based on the ingestion of 8 mg of the black plastic parts of sampled toys and A) HR-GCMS congener concentrations converted to apparent TEQ values, using TEF values for analogous chlorinated congeners of dioxins and furans; B) using DR CALUX and C) DR_{human} BEQ values. Weight-for-age values are based on WHO child growth standards.*

4. Conclusion

In plastic parts of selected sampled toys, we found high levels of PBDD/Fs and related TEQ levels as measured by GC-HRMS, and high dioxin-like activity as measured by the DR CALUX bioassay and its human variant DR_{human} CALUX. In three of the toys, GC-HRMS PBDD/F-related TEQ and CALUX activities were of similar or higher levels than the proposed limit value for toxic waste (> 1000 TEQ pg/g). Because PBDD/Fs are mostly generated during the process of fabrication of brominated flame retardants mixtures that are later added to plastics (Ren et al., 2011) and to a lesser extent during the recycling of such plastic (Ebert and Bahadir, 2003; Zhan et al., 2019) it means that the sampled toys very likely were manufactured using flame retardant-containing recycled plastics containing high levels of PBDD/Fs. This is a worrying finding considering that PBDD/Fs are potentially toxic chemicals that, at present, do not fall under EU regulation nor are there limit values for these compounds in consumer products, or other goods. It is necessary also to underline that current limits for both trace contamination and definition of POPs waste set in EU POPs Regulation (EU Regulation 2019/1021 of the European Parliament and of the Council of 20 June 2019 on persistent organic pollutants; Text with EEA relevance, 2019) for the total content of PBDEs are weak (1000 ppm) and will allow a large number of such contaminated products to enter the market (Petrlik et al., 2019).

Toxicity prediction and ranking of hazardous compounds are important issues in human hazard assessment and, because of the observed presence of high concentrations of PBDD/Fs in children's toys and observation that these compounds are being potent ligands for both the rodent and human AhR, more studies are needed towards the possible health-associated risks from exposure to PBDD/Fs from the usage of recycled plastics. Moreover, since the use of recycled plastics is stimulated globally, it is essential to monitor the contamination level of batches of recycling material before it is converted into renewed plastic use.

Finally, the here applied DR CALUX in vitro bioassays have identified an unknown risk of PBDD/Fs presence in PBDE pre-screened plastic products, which was confirmed later by analytical chemistry. Because of the challenges to be met by analytical chemistry, cost-efficient and high-capacity effect-based method such as bioassays are yet the only way forward to increase the globally needed capacities to test unknown, unexpected and various adverse chemicals contained in consumer products such as those made of recycled plastics.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary data

Material and Methods Supplementary Table 1 and Results and Discussion Supplementary Tables 2 and 3 are available (free of charges) on the article page of the publisher's website: https://doi.org/10.1016/j.chemosphere.2020.126579

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Chapter 4

Assessment of the effect of maternal smoking on placental and foetal hepatic AhR activity using a CALUX reporter gene assay with improved sensitivity, the DR_{hp} CALUX

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Abstract

The prenatal period is the most sensitive period with regards to environmental contaminant exposure and little is known about the biological effects elicited by chemicals and mixtures during pregnancy. Moreover, there is a need to identify methods which can be used to address the knowledge gap between *in-utero* exposure and observed adversities. From that perspective, bioassays allow making comprehensive analyses of the interaction of complex mixtures with biological pathways that are relevant during the development. In this study, we developed and used a high-performance (hp) AhR-responsive CALUX assay, the DR_{hp} CALUX, that was optimized for sensitive quantification of B[a]P-equivalent activity in small samples of foetal material (200mg) with a limit of detection of 0.007 ng of B[a]P-equivalent activity per gram of sample. We used the DR_{hp} CALUX assay to quantify the effect of maternal smoking on the activity of the aryl hydrocarbon receptor (AhR), a major biological target of the PAHs contained in cigarette smoke, in the placenta and foetal liver at the late first/early second trimester of normal pregnancy. Our main finding was that maternal smoking resulted in a significant 4-fold increase of PAH-mediated AhR activity in the placenta of smoking mothers as compared to non-smoking mothers. There was no significant difference between foetal livers that were exposed or not to maternal smoking. This suggests that the PAHs contained in cigarette smoke may significantly affect AhR-signalling in the placenta and therefore that the disruption of AhR-signalling may be an important component in the initiation of adverse developmental outcomes induced by PAH exposure.

1. Introduction

There is a great need to understand the complex interactions between exposure to exogenous chemicals and the aetiology of major human diseases. While there is increased understanding in the molecular pathways involved, the complexity of the mixtures of contaminants hampers the possibilities to find correlative associations with disease outcomes. Besides, only a limited number of chemicals are routinely analysed as a proxy to represent the complex mixture of contaminants from food and environmental sources humans are exposed to. The increased knowledge of major biomolecular pathways through which toxic compounds can act in conjunction opens opportunities to make comprehensive analyses on complex mixture interactions with those pathways and their role in disease aetiology.

Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous contaminants with detrimental effects on human health (Edwards Susan Claire et al., 2010; Kim et al., 2013; F. Perera et al., 2005). The prenatal period is considered the most sensitive period for PAHs exposure and exposure is associated with post-natal deleterious effects (Edwards Susan Claire et al., 2010; F. Perera et al., 2005; Tang et al., 2014). The adverse effects of PAH-containing mixtures on the developing foetus have been reported in human epidemiological studies where *in-utero* exposure to PAHs via maternal smoking or urban pollution appeared to be linked with various adverse developmental and reproductive outcomes in the offspring such as low birth weight and size, preterm birth and reduced fertility (Dechanet et al., 2011; Edwards Susan Claire et al., 2010; Fowler et al., 2011, 2008; Kleinman et al., 1988; F. Perera et al., 2005). Because of the chemical complexity of cigarette smoke, the role of the PAHs contained in cigarette smoke in adversities related to maternal smoking remain uncertain (O'Shaughnessy et al., 2011).

There are hundreds of different PAH congeners and over 500 PAHs have been identified in cigarette smoke (Rodgman and Perfetti, 2006). So far, however, epidemiological studies on the effects of PAHs contained in cigarette smoke have focused on the chemical-analytical determination of very limited sets of PAH congeners only, e.g. the so-called EPA-16 (Keith, 2015), which is a selection of ubiquitous PAHs, that are not necessarily the most toxic/potent ones (Pieterse et al., 2013). In addition, analytical chemistry-based determination of PAHs does not provide information on and cannot predict the overall effects of complex mixtures of PAHs, which is necessary to evaluate its toxicity. Thus, more comprehensive methods should be employed to determine the role of maternal smoking-derived PAH mixtures on adverse developmental effects, and on the biological pathways involved.

In the case of PAHs, a major target is the aryl hydrocarbon receptor (AhR; Denison and Nagy, 2003; Machala et al., 2001). The AhR is a ligand-activated transcription factor involved in xenobiotic sensing and metabolism, as well as in many developmental processes including haematopoiesis, liver cell development and development of the immune system (Larigot et al., 2018). During foetal life, the AhR is highly expressed in organs such as the liver and placenta (Jiang et al., 2010; Yamamoto et al., 2004), which

suggests an important role for the receptor in these organs. Moreover, the adverse health effects related to prenatal PAHs exposure are reminiscent to those elicited by dioxins whose toxicity is also mediated by the AhR. This indicates that the AhR likely plays an important role in the PAH-mediated adverse health outcomes associated with maternal smoking (Ahlborg et al., 1992; Birnbaum, 1995; Brouwer et al., 1995).

The pivotal role of AhR in the mode-of-action of dioxins and PAHs prompted us to develop quantitative AhR-based cellular bioassays as an alternative and/or complement to traditional analytical-chemical-based methods and can provide relevant information about the mixture toxicity of PAHs (Murk et al., 1996; Pieterse et al., 2013). In cell-based AhR-responsive quantitative reporter gene assays such as chemically activated luciferase (CALUX) bioassays, the induction of the luciferase transcription occurs via the interaction of a PAH single compound, or mixture-activating the Ah-receptor pathway and is dose-dependent. Therefore, it is possible to directly determine the total AhR-activity of a sample and express it as bioanalytical B[a]P-equivalent (B[a]P-eq) expressed as, e.g. nanograms B[a]P-eq per gram of tissue. An advantage of cell-based assays as compared to analytical chemistry is that they provide information on the total bioactivity of a mixture which comprises the activity of all individual PAH congeners on the AhR and is not limited to a handful of selected congeners.

In this study, we used a specifically developed high performance (hp) version of the DR CALUX bioassay, the DR_{hp} CALUX, to quantify the effect of maternal smoking on PAH-mediated AhR activity in placentas and livers from foetuses obtained from electively terminated, normally progressing pregnancies (late first/early second trimester) of smoking and non-smoking mothers. To our knowledge, this is the first study that quantifies the effect of maternal smoking on the AhR, a highly relevant biomolecular pathway in reproduction and development, in the placenta and foetal liver.

2. Material and Methods

2.1. Samples

The collection of foetal material was approved by the British National Health Service Grampian Research Ethics Committee (REC 04/S0802/21). Women over 16 years of age, seeking elective terminations of pregnancy were recruited with written informed consent. Foetal liver and placenta were collected from normally progressing pregnancies ranging between 11 to 21 weeks of gestation. Six full-thickness biopsies were taken from each placental sample and were then combined and pulverized in liquid nitrogen to obtain a representative powder. Foetal livers were removed and weighed. All tissues were stored at -80^{o}C until analysis. Information about smoking status was initially collected directly from the mother via questionnaire and was confirmed by cotinine concentration determination. Cotinine is a metabolite of nicotine that is used as a marker of smoking. Levels of cotinine in the samples were determined with a commercial kit as described previously (Fowler et al., 2008).

2.2. Chemicals

2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) was purchased as an analytical standard from Wellington Laboratories (Guelph, Canada). B[a]P was purchased as an analytical standard from Sigma Aldrich (Darmstadt, Germany). Dimethyl Sulfoxide (DMSO) was obtained from Acros organics (Geel, Belgium) and n-hexane, Isopropanol and HPLC-grade water were purchased from BioSolve (Valkenswaard, The Netherlands).

2.3. Cell-culture media

Cell culture medium (maintenance and assay media) consisted of alpha-MEM (Gibco) medium supplemented with 10% foetal calf serum (FCS), 10% non-essential amino acids and streptomycin (10mg/mL) plus penicillin (10U/mL). To reduce background AhR activity when using short (4 hours) incubation times conditioned medium was used consisting of filtered culture medium used to culture DR_{hp} CALUX cells for at least 48 hours (Pieterse et al., 2013).

2.4. Cell lines and cell culture

DR and DR_{hp} CALUX cells were cultured in maintenance medium in a humidified atmosphere at 37^{o}C and 5% CO_2 and cultured twice a week at a ratio of 1:10. The DR_{hp} CALUX cell line consists of wild-type rat hepatoma H4IIE cells stably transfected with an AhR-responsive plasmid. The AhR-construct expressed in the DR_{hp} CALUX cell line is a variant of the construct expressed by the DR CALUX cell line (Garrison et al., 1996) and consists of a tandem of 5 times the 484 bp dioxin-responsive domain cloned inserted in the BglII site of the pGudLuc1 plasmid resulting in a pGudLuc1.5xmcyp1a1 reporter plasmid with a total of 20 dioxin-response elements (DREs; Van Vugt-Lussenburg et al., 2010). To obtain the DR_{hp} CALUX cell line, stable transfection of H4IIE cells was achieved using the Lipofectamine 2000 method according to the manufacturer's instructions (Invitrogen, CA – USA). H4IIE wild-type cells were transfected with both pGudLuc1.5mcyp1a1 plasmid and geneticin resistance plasmid pSG5-neo (Sonneveld et al., 1998). The geneticin concentration used for antibiotic resistance selection of H4IIe transfectants was determined before the transfection and equal to 275 µg/mL. After antibiotic and clonal selection, the stability of the final transfectant, referred to as DR_{hp} CALUX, was evaluated in continuous culture and cells were exposed to 2,3,7,8-TCDD over an extended period. The DR_{hp} CALUX cells were found to be stable and highly inducible, up to 90 passages, which is 45 weeks of continuous culture.

2.5. CALUX analysis of pure compounds and samples

DR_{hp} CALUX cells from continuous cultures were resuspended in assay medium to a density of 300,000 cells/mL. Using an automatic multi-channel pipette, 100 uL of the cell suspension was distributed over a transparent 96-wells plate, excluding the outer wells, and the plates placed in an incubator (a humidified atmosphere at 37°C and 5% CO_2). After 24 hours sub-confluent cells were retrieved, and the exposure medium was prepared. The exposure medium was prepared manually using either assay medium or

conditioned medium (section cell-culture media) depending on the duration of incubation. For the analysis of pure compounds, a 9-step dilution series in 1 log unit increments of the analysed compound (dissolved in DMSO) was prepared in the medium corresponding to the exposure time. On each plate, a complete reference curve of 2,3,7,8-TCDD (24 hours analysis of pure compounds) or B[a]P (2 hours to 24 hours analysis of pure compounds and samples) dilution series was analysed. In the case of sample analysis, 5-step dilution series in 1 log unit increments of the analysed sample was prepared in conditioned medium (4 hours analysis, B[a]P reference) and on each plate. After the exposure, the medium was discarded, and cells were lysed using Triton lysis buffer and the plates mixed on a shaker for approximatively 5 minutes. The luciferase activity was measured using a luminometer (Berthold, Bad Wildbad, Germany) after the addition of the luciferin-containing solution.

2.6. Preparation of reference material and B[a]P-fortified reference material

Reference material was not available for the investigated matrices. Therefore, pig liver was used as reference material as its anatomy as well as physiology is comparable to that of the human liver (Cooper et al., 2016; Junatas et al., 2017). An entire frozen pig liver purchased at a local butcher shop was homogenized to prepare B[a]P-fortified and control aliquots of approximatively 200mg (wet weight). The B[a]P-fortified aliquots were spiked at least 24 hours before the extraction.

2.7. Sample extraction

Aliquots of liver (137–356mg wet weight) and placenta (174–295mg wet weight) were homogenized in 1 volume of HPLC-grade water (5mL) in a hexane-cleaned glass tube. After vigorous mixing, 1 volume of isopropanol and 2 volumes of hexane were added to the sample, the phases separated by shaking for at least 15min and the upper n-hexane phase was transferred to another hexane cleaned glass tube. Samples were extracted twice again. The final hexane extracts were evaporated under a gentle stream of N_2 and the fat content was determined by gravimetric analysis before the addition of 1mL hexane. The 1mL concentrated extracts were then transferred to a conical vial for further evaporation and transfer to 25µL of DMSO. The samples were randomized and extracted in 9 batches. In total, 44 placenta samples and 43 foetal liver samples of approximatively 200mg were extracted. The median amount of fat extracted from each sample was 2.05mg (0.1–3.8mg; 0.8%) for the placenta and 3.4mg (0.30–10.3mg; 1.5%) for the liver.

2.8. Determination of CALUX bioanalytical-equivalents, quality control

Each sample was analysed in triplicate. On each 96-well plate, a complete 10-points B[a]P concentration range was analysed in triplicate and the rest of the plate, excluding the outer wells was used to analyse a dilution range of two samples. For every 96-well plate several parameters were checked to verify the validity of the results: (i) R2 of standard curve >0.98, (ii) z-factor of standard curve >0.6, (iii) B[a]P EC_{50} between the assay-specific pre-determined limit values and (iv) SD of analysed triplicates <15%. Then, relative light units from luminescence determination were interpolated from the B[a]P

standard dose-response curve of the plate to determine the CALUX B[a]P-equivalent activity (B[a]P-eq) expressed as ng B[a]P-eq/g tissue. The CALUX activity was quantified between the limit of quantification (LOQ) and the EC_{50} of B[a]P and, as close as possible to the EC_{10} of B[a]P (Besselink et al., 2004). A sample was considered active when the B[a]P-eq was higher than the LOQ. Batch-specific extraction efficiency was determined by extracting, for each batch, a reference material sample spiked with B[a]P and a non-spiked reference sample. The average apparent recovery for B[a]P in the reference material was 71%. A procedure blank was also extracted in each batch. Batches in which the activity of the non-spiked reference material significantly deviated from the average value of the non-spiked reference were excluded from the analysis.

2.9. Data analysis and performance determination

The software GraphPad Prism was used for dose-response modelling employing four-parameters nonlinear regression model (1).

$$(Y = Bottom \ + \ (Top - Bottom)/(1 + 10\hat{}((LogEC50 - X) * HillSlope) \qquad (1)$$

Using raw luminescence values (relative light units, RLU), the maximal response of the reference compound was set at 100% and the response of the analysed compound or sample was expressed relative to the maximum response of the reference compound. Values presented in graphs are presented as mean ± standard deviation (SD).

The reproducibility and stability of the analysis were determined using the coefficient of variation (%CV) and the coefficient of reproducibility ($\%VC_R$) which were calculated based on the EC_{50} value and from log-transformed luminescence data using the equations (2) and (3).

$$\%CV = \ 100 * (\%SD/Mean) \qquad (2)$$

$$\%VC_R = \ 100 * \sqrt{\frac{\sum_{i=1}^{n} (\frac{EC50_{i,1} - EC50_{i,2}}{0{,}5(EC50_{i,1} + EC50_{i,2})})^2}{2n}} \qquad (3)$$

$EC50x_{i,1}$: log (EC_{50}) i^{th} determination first observation

$EC50x_{i,2}$: log (EC_{50}) i^{th} determination second observation

n : number of determinations

The limit of detection (LOD) was determined from raw luminescence data using equation (4) and the limit of quantification (LOQ) is equal to 3 times the LOD.

$$LOD = Mean_{blank} + (3 \times SD_{blank}) \qquad (4)$$

Statistical analyses were performed using GraphPad Prism built-in functions. The normality of data distribution was tested with the Shapiro-Wilk test and non-normally distributed data were log-transformed (log10) and the normality retested prior to

statistical analysis. Control and smoke-exposed groups were compared using the independent t-test. Results were deemed to be significant when the P-value was <0.05.

3. Results and discussion

3.1. Optimization of the DR_{hp} CALUX bioassay for the determination of B[a]P-equivalent activity

In the DR_{hp} CALUX cell line, the luciferase reporter gene is under the control of 5 times more DREs than in the DR and PAH CALUX H4IIE based AhR-responsive cell lines (Garrison et al., 1996; Pieterse et al., 2013) whereas the rest of the reporter construction is identical to the one expressed in the DR CALUX. A comparison of the DR and DR_{hp} CALUX bioassays based on 10 independent 2,3,7,8-TCDD dose-response determinations (Table 1) indicated that the DR_{hp} CALUX cells were 4-fold more inducible by saturating concentrations of 2,3,7,8-TCDD and 5-fold more sensitive than DR CALUX based on a limit of detection of 5E-13M for 2,3,7,8-TCDD. Therefore, although the cell line was applied here for the determination of B[a]P-equivalent activity, DR_{hp} CALUX cells also appear to be potentially relevant for sensitive quantification of dioxins and dioxin-like compounds using the DR CALUX® method.

We next determined the responsiveness and sensitivity of DR_{hp} CALUX cells to benzo[a]pyrene (B[a]P), an established human carcinogen and generally used model compound for PAHs acting through the AhR (Pieterse et al., 2013). Since PAHs are metabolised in the metabolically active H4IIE cells their response is incubation time-dependent (Pieterse et al., 2013), therefore we evaluated the optimal incubation time for the detection of PAH-mediated activity in DR_{hp} CALUX cells. For this, we exposed DR_{hp} CALUX cells to a full dose-response of B[a]P and measured B[a]P-mediated AhR activity at time points ranging from 1 to 24 hours (1, 2, 4, 6 8, 16, 20 and 24 hours; Figure 1). As shown in Figure 1 we observed a time-dependent increase in induction factor that reaches a peak at 4 hours of incubation followed by a decrease until 24 hours. This confirms that the metabolism of B[a]P by the cells results in a loss of ligand-dependent induction over time. Therefore, we decided to use a 4-hours incubation period for the determination of PAH-mediated activity in the samples.

Table 1. *Comparison of the DR_{hp} and DR CALUX bioassays based on the EC_{50} for 2,3,7,8-TCDD, limit of detection, luciferase activity and fold induction.*

Assay	EC_{50} (M)	LOD (M)	Background luciferase activity (RLU ÷ 10 000)	Maximal luciferase activity (RLU ÷ 10 000)	Maximal fold induction
DR_{hp} CALUX (n=10)	2.4E-12	1.0E-13	0.4	24.2	57
DR CALUX (n=10)	6.5E-12	5.0E-13	2.1	32.6	14

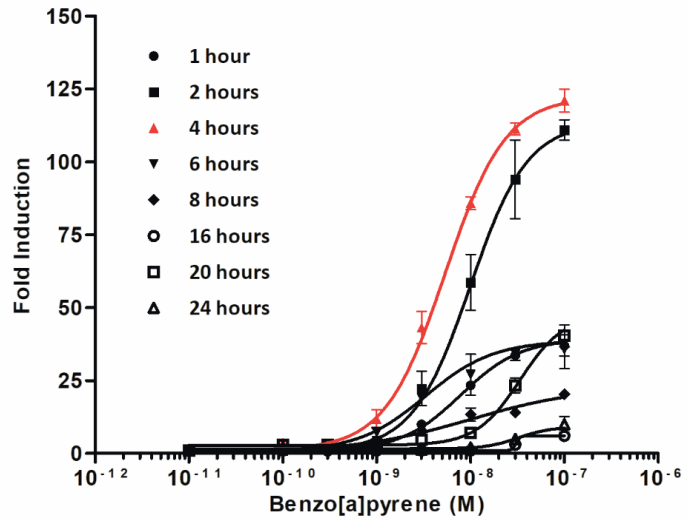

Figure 1. *Dose-response of DR_{hp} CALUX cells exposed to Benzo[a]pyrene for different incubation periods ranging from 1h to 24h (mean ± SD).*

Since we intended to use the bioassay quantitatively, we further determined the performances of the bioassay for quantitative analysis. To that end, we determined the limit of detection (LOD) and limit of quantitation (LOQ) values of the bioassay as well as its reliability and reproducibility via the determination of the coefficient of variation ($\%CV_{EC50}$) and coefficient of reproducibility ($\%VCR_{EC50}$) both based on the EC_{50} values of B[a]P. The values for the $\%CV_{EC50}$ and $\%VCR_{EC50}$, were 1.45% and 2.11%, respectively with an EC_{50} equal to 5.9E-10M. The LOD for the B[a]P reference was 8.9E-12M (mol per litre of medium) that is equivalent to 0.007 ng of B[a]P per gram of sample and the LOQ 2.68E-11M that is equivalent to 0.021 ng of B[a]P per gram of sample.

Our next step was to determine if the DR_{hp} CALUX was sensitive enough to determine the PAH-related bioactivity in small amounts of foetal liver samples. To do so, we estimated the B[a]P-equivalent bioactivity in foetal liver previously analysed for the 16-EPA PAHs by analytical chemical methods (Fowler et al., 2014, 2008). To express chemical-based concentrations as biological equivalents we multiplied individual PAH congener concentrations by their respective relative potency (REP) values previously determined in PAH CALUX (H4IIE cells, Pieterse et al., 2013); then summed individual values to obtain the total bioanalytical equivalent expressed as ng B[a]P-eq./g of tissue (Supplementary data).

The lowest B[a]P-equivalent activity estimation was observed in the livers from non-exposed female foetuses (Fowler et al., 2014) and equal to 7.8 ng B[a]P-equivalent/g, which is considerably higher than the LOQ value (LOQ = 0.11 ng B[a]P-equivalent for 200mg of sample) for PAH determination using the DR_{hp} CALUX method. This meant that the quantification of PAH-mediated activity in the small amounts of foetal sample (200mg

of sample available) was possible. Although unrealistic in terms of sample handling, the lowest theoretical amount from which it would have been possible to quantify PAH-mediated activity in the female foetal liver of this study is 2.6 mg. It should be noted that all the estimations of minimal sample volume required considering the volume needed for analysing, re-analysing, and preparing dilutions of the sample.

3.2. DR_{hp} CALUX analysis of placenta and foetal liver samples

After optimization of the DR_{hp} CALUX bioassay for the determination of B[a]P-equivalent activity in foetal samples, we proceeded with the processing and analysis of the human foetal liver and placenta samples. A total of 30 foetal liver and 29 placenta samples were extracted and analysed using the DR_{hp} CALUX bioassay, a summary of the results is presented in Table 2 and 3, respectively.

A general observation was that, in the samples from non-smoking mothers, the B[a]P-equivalent activity level was in the same range for both placenta and foetal liver (Table 2 and 3). However, in the placenta samples, we found that the average level of B[a]P-equivalent activity from smoking mothers was statistically different and about 4-fold higher than the average level in placentas from the non-smoking controls (Table 2). Figure 2 represents a line-up of the B[a]P equivalence values for the individual placenta samples of controls and smoking mothers, showing the range and differences in B[a]P-equivalence levels between smokers and non-smokers. The elevated B[a]P-equivalent activity observed in the placenta of smoking mothers is in line with the increase of CYP1A1 transcription and catalytic activity observed in the placenta from smoking mothers by Huuskonen and co-workers (Huuskonen et al., 2016). When placenta groups were divided into genders, a difference between male and female control placenta groups was observed based on the mean values (Table 2) whereas the mean values were similar for smoke-exposed groups. Given the small size of the subgroups, it is likely that this difference is attributed to random statistical distribution however, a biological cause may be possible as well. Indeed, the placenta shows sex-dependent structural and functional differences which may then be translated in differences in background AhR-activity (Clifton, 2010; Pérez-Cerezales et al., 2018; Rosenfeld, 2015). A study on a larger group of samples is recommended to investigate the reason behind the observed sex-specific-differences in background AhR activity in the foetal placenta. Nonetheless, there was a 2-fold difference (not statistically significant) between male from the control group and male placenta from smoking mothers.

In the case of the foetal liver samples, there was no statistically significant difference in average B[a]P-equivalent activities between foetuses from non-smoking controls and smoking mothers, although the average B[a]P-equivalent activity appeared to be lower in the samples from smoking mothers (Table 3). This absence of a marked difference in foetal liver B[a]P-equivalent levels between smokers and non-smoking mothers is in line with earlier studies (Fowler et al., 2014, 2008) who measured analytical chemistry-based PAH concentrations. In the liver, differences in gender within groups were not observed.

Table 2. *Summary of bioanalytical B[a]P-equivalent activity in placenta samples measured with DR_{hp} CALUX*

	Control (n=13)			Smoke-exposed (n=16)			
	Mean BEQ ng B[a]P-eq./g	**SD**	**Range**	**Mean BEQ ng B[a]P-eq./g**	**SD**	**Range**	**P-value***
Total (n=29)	4.45 (n=13)	6.0	0.2-20.7	17.80 (n=16)	19.8	2.5-60.2	**0.0232**
Male (n=14)	10.77 (n=6)	6.2	1.4-20.7	21.13 (n=8)	21.9	2.9-60.2	0.5947
Female (n=15)	3.02 (n=7)	2.7	0.2-8.9	14.46 (n=8)	16.5	2.5-57.4	**0.0096**

*: statistically significant P-values are shown in bold underlined script

Table 3. *Summary of bioanalytical B[a]P-equivalent activity in foetal liver samples measured with DR_{hp} CALUX.*

	Control (n=13)			Smoke-exposed (n=16)			
	Mean BEQ ng B[a]P-eq./g	**SD**	**Range**	**Mean BEQ ng B[a]P-eq./g**	**SD**	**Range**	**P-value***
Total (n=30)	3.28 (n=13)	3.2	0.3-11.8	2.92 (n=17)	4.9	0.2-21.3	0.0986
Male (n=16)	3.76 (n=7)	3.9	0.3-11.8	1.33 (n=9)	0.9	0.3-2.8	0.0815
Female (n=14)	3.64 (n=6)	2.1	0.9-6.1	4.72 (n=8)	6.63	0.2-21.3	0.2964

*: statistically significant P-values are shown in bold underlined script

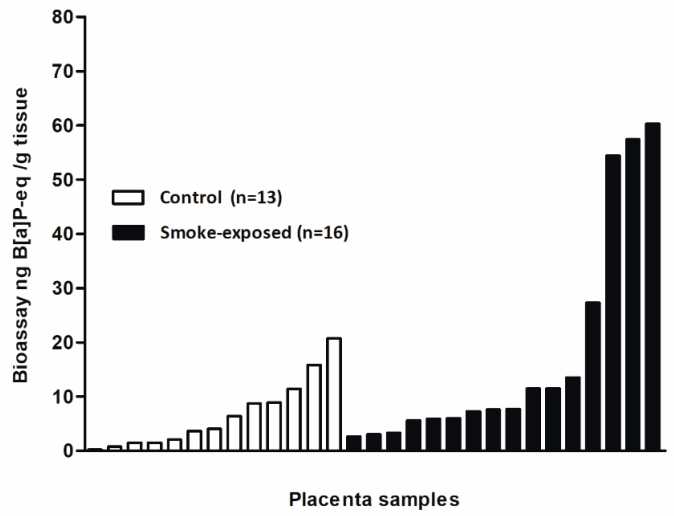

Figure 2: *B[a]P-equivalent activity per individual placenta sample from smokers (n = 16) and non-smokers (n = 13) measured with the DR_{hp} CALUX and expressed as ng B[a]P-equivalent activity per gram of tissue.*

Overall, our results show that maternal smoking increases B[a]P-equivalent activity in the placenta but not in the foetal liver of the offspring. Because in the CALUX bioassay, elevated B[a]P-equivalent activity is directly correlated with elevated AhR-transactivational activity, our results also suggest that maternal smoking may significantly activate AhR-signalling in the placenta. Since the AhR fulfils essential functions in the placenta besides xenobiotic sensing, the disruption of AhR-signalling by the PAHs contained in cigarette smoke may be an important component in the initiation of certain maternal-smoking induced adverse health effects, such as low birth weight (Choi et al., 2003; Perera Frederica et al., 2004) and neurobehavioral effects (Perera Frederica P. et al., 2012). Our results also suggest that the fraction of AhR-interacting PAHs which can cross the placenta has a lesser effect on AhR activity in the liver of the offspring, possibly due to clearance by placental CYP450 enzymes and potential further metabolization by foetal hepatic enzymes (Hakkola et al., 1998; Myllynen et al., 2005). However, the adverse effects of maternal smoking on the offspring are known (Dechanet et al., 2011; Fowler et al., 2011, 2008; Kleinman et al., 1988), and it should be emphasized that the CALUX activity only reports B[a]P-equivalent activity as the total effect of AhR-interacting PAHs. Indeed, there are PAHs and PAHs metabolites that do not interact with the Ah-receptor and may have effects on other critical pathways and biological events. Further investigations, targeting relevant biological pathways other than the AhR, are necessary to better comprehend the effects of the PAH-mixture to which foetuses are exposed via maternal smoking.

4. Conclusion

In this study, we quantified the effect of maternal smoking on PAH-mediated AhR activity in placenta and liver from foetuses of (non-)smoking mothers. To that end, we used a sensitive CALUX bioassay for the determination of CALUX B[a]P-equivalent activity. The enhanced high performance (hp) DR_{hp} CALUX bioassay used in this study was capable of very sensitive detection of B[a]P-equivalent with excellent reliability. The sensitivity of the CALUX bioassay employed in this study is relevant in the case of epidemiological studies, particularly those focusing on foetal material where only small amounts of sample are available. Furthermore, the sensitivity of the DR_{hp} CALUX bioassay is relevant also in the context of the regulatory control and monitoring of PAHs (and dioxins) in food and feed. Indeed, a sensitive bioassay with low limits of detection/quantification allows using a reduced volume of sample which directly implies that the volume of consumables, such as solvents, can be reduced as well as the associated analytical costs. The CALUX analysis of B[a]P-equivalent activity in the foetal samples indicated that maternal smoking resulted in a 4-fold elevation of B[a]P-equivalent activity in the placenta compared to the placenta of non-smoking mothers. This suggests that the PAHs contained in cigarette smoke may significantly affect AhR-signalling in the placenta. We found no significant difference in B[a]P-equivalent activity between the foetal livers of smoking and non-smoking mothers, which suggest that lower concentrations of AhR-interacting PAHs may reach the foetus. Our results may have important implications for understanding the molecular basis for observed smoking-related adverse health effects

in human infants and emphasize that the AhR-signalling disruption *in utero* may play an important role in the prenatal initiation of (human) adverse effects. Finally, and as illustrated in this study, cell-based bioassays are relevant to use in the context of epidemiological studies as they enable to study the integrated effect of complex mixtures on biological relevant pathways.

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Conflicts of interest

The authors declare no conflict of interest.

Supplementary data

Supplementary data. *B[a]P-equivalent bioactivity in foetal liver derived from chemically determined concentrations of PAHs*

	Control ng B[a]P-eq/g	Smoke-exposed ng B[a]P-eq/g
Male foetal liver (Fowler et al. 2008)	130.5	86.3
Female foetal liver (Fowler et al. 2014)	7.8	10.9

Relative potencies (REP) values for PAHs determined by Pierterse et al (2004) in H4IIE cells:

Congener	Molecular weight	REP Pieterse et al. (2013)
	Mw (g/mol)	(mol/mol)
Naphtalene	128	<0.0001
Acenaphtalene	152	<0.0001
Acenaphtene	154	<0.0001
Fluorene	166	<0.0001
Phenantrene	178	<0.0001
Anthracene	178	<0.0001
Fluoranthene	202	<0.0001
Pyrene	202	<0.0001
Benzo[g,h,i]perylene	276	<0.0001
Benzo[a]anthracene	228	0.3
Chrysene	228	0.8
Benzo[a]pyrene	252	1
Dibenzo[a,h]anthracene	278	1.3
Indeno[1,2,3-cd]pyrene	276	1.3
Benzo[k]fluoranthene	252	3.7
Benzo[b]fluoranthene	252	5

Evaluation of the B[a]P equivalent activity in male foetal liver (Fowler et al., 2008):

Congener	Control		
	ug/kg	mol/g	mol B[a]P-eq/g
Naphtalene	98	7.7E-10	nc
Acenaphtalene	52	3.4E-10	nc
Acenaphtene	100	6.5E-10	nc
Fluorene	159	9.6E-10	nc
Phenantrene	382	2.1E-09	nc
Anthracene	18	1.0E-10	nc
Fluoranthene	na	nc	nc
Pyrene	na	nc	nc
Benzo[g,h,i]perylene	0.8	2.9E-12	nc
Benzo[a]anthracene	331	1.5E-09	4.4E-10
Chrysene	11	4.8E-11	3.9E-11
Benzo[a]pyrene	0.8	3.2E-12	3.2E-12
Dibenzo[a,h]anthracene	0.8	2.9E-12	3.7E-12
Indeno[1,2,3-cd]pyrene	0.8	2.9E-12	3.8E-12
Benzo[k]fluoranthene	0.5	2.0E-12	7.3E-12
Benzo[b]fluoranthene	1.3	5.2E-12	2.6E-11
Total ug/kg	1156		
Total mol B[a]P-equivalent/g			5.2E-10
Total ng B[a]P-equivalent/g			130.52

nd: not determined; nc: not calculated

Congener	Smoke-exposed		
	ug/kg	mol/g	mol B[a]P-eq/g
Naphtalene	152	1.2E-09	nc
Acenaphtalene	56	3.7E-10	nc
Acenaphtene	71	4.6E-10	nc
Fluorene	147	8.9E-10	nc
Phenantrene	175	9.8E-10	nc
Anthracene	19	1.1E-10	nc
Fluoranthene	nd	nc	nc
Pyrene	nd	nc	nc
Benzo[g,h,i]perylene	0.2	7.2E-13	nc
Benzo[a]anthracene	195	8.6E-10	2.6E-10
Chrysene	9	3.9E-11	3.2E-11
Benzo[a]pyrene	0.2	7.9E-13	7.9E-13
Dibenzo[a,h]anthracene	0.2	7.2E-13	9.4E-13
Indeno[1,2,3-cd]pyrene	0.5	1.8E-12	2.4E-12
Benzo[k]fluoranthene	1	4.0E-12	1.5E-11
Benzo[b]fluoranthene	1.8	7.1E-12	3.6E-11
Total ug/kg	827.9		
Total mol B[a]P-equivalent/g			3.4E-10
Total ng B[a]P-equivalent/g			86.34

nd: not determined; nc: not calculated

Evaluation of the B[a]P equivalent activity in female foetal liver (Fowler et al., 2014)

Congener	Control		
	ug/kg	mol/g	mol B[a]P-eq/g
Naphtalene	7.44	5.8E-11	nc
Acenaphtalene	0.45	3.0E-12	nc
Acenaphtene	0.16	1.0E-12	nc
Fluorene	6.48	3.9E-11	nc
Phenantrene	13.7	7.7E-11	nc
Anthracene	1.32	7.4E-12	nc
Fluoranthene	1.73	nc	nc
Pyrene	<LOD	nc	nc
Benzo[g,h,i]perylene	0.46	1.7E-12	nc
Benzo[a]anthracene	1.42	6.2E-12	1.9E-12
Chrysene	3.56	1.6E-11	1.2E-11
Benzo[a]pyrene	<LOD	nc	nc
Dibenzo[a,h]anthracene	nd	nd	nd
Indeno[1,2,3-cd]pyrene	nd	nd	nd
Benzo[k]fluoranthene	<LOD	nc	nc
Benzo[b]fluoranthene	0.83	3.3E-12	1.6E-11
Total ug/kg	37.55		
Total mol B[a]P-equivalent/g			3.1E-11
Total ng B[a]P-equivalent/g			7.77

<LOD: below limit of detection; nd: not determined; nc: not calculated

Congener	Smoke-exposed		
	ug/kg	mol/g	mol B[a]P-eq/g
Naphtalene	16.9	1.3E-10	nc
Acenaphtalene	<LOD	nd	nc
Acenaphtene	5.67	3.7E-11	nc
Fluorene	72.8	4.4E-10	nc
Phenantrene	89.1	5.0E-10	nc
Anthracene	19	1.1E-10	nc
Fluoranthene	7.29	nc	nc
Pyrene	11.37	nc	nc
Benzo[g,h,i]perylene	0.67	2.4E-12	nc
Benzo[a]anthracene	1.43	6.3E-12	1.9E-12
Chrysene	0.01	4.4E-14	3.5E-14
Benzo[a]pyrene	1.37	5.4E-12	5.4E-12
Dibenzo[a,h]anthracene	nd	nc	nc
Indeno[1,2,3-cd]pyrene	nd	nc	nc
Benzo[k]fluoranthene	1	4.0E-12	1.5E-11
Benzo[b]fluoranthene	1.07	4.2E-12	2.1E-11
Total ug/kg	227.68		
Total mol B[a]P-equivalent/g			4.3E-11
Total ng B[a]P-equivalent/g			10.90

<LOD: below limit of detection; nd: not determined; nc: not calculated

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Chapter 5

Versicolorin A enhances the genotoxicity of Aflatoxin B1 in human liver cells by inducing the transactivation of the Ah-Receptor

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Abstract

Mycotoxins are food contaminants that have major adverse effects on human health. The mycotoxin aflatoxin B1 (AFB1) is the most important aflatoxin, as it is a proven human hepatotoxin and a potent carcinogen when converted into a DNA reactive form by cytochrome P450 enzymes (CYP450). The biosynthesis of AFB1 involves multiple enzymatic steps and precursors. Of these, Versicolorin A (VerA) shares structural similarities with AFB1, which suggest similar toxicological effects. Moreover, VerA can be found in significant amount in contaminated commodities, often co-occurring with AFB1. This study investigated and compared the cytotoxic and genotoxic effects of AFB1 and VerA alone or in combination in HepG2 human liver cells. Our results show that AFB1 and VerA have similar cytotoxic effects and are both genotoxic. However, AFB1 and VerA seem to have different genotoxicity mechanisms. Unlike AFB1, the main mechanism of VerA does not involve the formation of DNA double-strand breaks by DNA-reactive metabolites. The results also show that VerA can activate the aryl hydrocarbon receptor (AhR) and significantly induce the expression of the cytochrome P450 1A1 (CYP1A1) while AFB1 did not induce AhR-dependent CYP1A1 activation. The combination of VerA with AFB1 resulted in enhanced genotoxic effects, suggesting that activation of the AhR by VerA can influence AFB1 genotoxicity by promoting its bioactivation by CYP450s to a highly DNA-reactive metabolite. The present results emphasize the need for expanding the toxicological knowledge regarding mycotoxin biosynthetic precursors to identify those who may pose, directly or indirectly, a threat to human health.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by moulds that may contaminate food commodities. Aflatoxins are a group of mycotoxins synthesized mainly by moulds from *Aspergillus flavus* and *A. parasiticus* species which can contaminate crops such as maize, rice and nuts, typically in tropical and sub-tropical areas (Schrenk et al., 2020). Among the aflatoxins, aflatoxin B1 (AFB1) has received particular attention due to its high carcinogenic potential for humans (International Agency for Research on Cancer 1976; Ostry et al., 2017). The health issues related to the presence of AFB1 in food commodities are major and include carcinogenicity, impaired development, immunotoxicity, and even death in case of severe acute exposure (Meissonnier et al., 2008; Schrenk et al., 2020). Consequently, its presence is monitored and regulated in food and feed in Europe (Commission Regulation (EC) No 1881/2006) and other parts of the world.

The biosynthesis of AFB1 involves multiple enzymatic steps and the accumulation of different precursor molecules. Versicolorin A (VerA) is a key precursor as its accumulation is an important parameter for triggering the final steps of AFB1 synthesis, however, it has been reported that the conversion of VerA to AFB1 is not complete in certain *Aspergillus* species (Conradt et al., 2015; Lee et al., 1976). Therefore, both mycotoxins can co-occur and VerA can even be detected at a higher concentration than AFB1 in certain food commodities (Abdallah et al., 2017; Abia et al., 2013; Janić Hajnal et al., 2020). The data on the toxicity of VerA are limited, but suggest a high toxic potency of this molecule to humans, with the induction of severe genotoxicity and cytotoxicity in different human cell lines (Gauthier et al., 2020; Jakšić et al., 2012; Theumer et al., 2018). Because both molecules can co-contaminate food commodities, comparative toxicity studies between AFB1 and VerA are needed to determine if the latter may pose a threat to human health. Moreover, it is of interest to assess if combined toxicity can occur, particularly in organs relevant to AFB1's toxicity such as the liver.

From a chemical-structural point of view, AFB1 is characterized by having a furofuran-ring with a double bond at the 8,9-position (Supplementary Figure 1). This double bond is converted *in vivo* into the highly reactive exo-AFB1-8,9-epoxide (AFBO). The bioactivation of AFB1 to AFBO is mediated by cytochrome p450 enzymes (CYP450s), mainly in the liver. The reactive AFBO is responsible for covalent DNA and protein binding that can lead to DNA mutations and cytotoxicity. Several human epidemiological studies have identified a strong mechanistic link between exposure to AFB1, its genotoxic effects and, a mutation in a specific codon of the p53 tumour-suppressor gene, which abrogates the function of the tumour repressor and contributes to the progression to hepatocellular carcinoma (Jackson et al., 2003; Stern et al., 2001). Alike AFB1, VerA features a double bond in its final furofuran ring (Supplementary data 1), but it is unclear if its genotoxic effects are dependent on its bioactivation by CYP450s (Gauthier et al., 2020). CYP450s are a large family of enzymes involved in the biotransformation of xenobiotics and endogenous chemicals. The abundance and activity of CYP450s are

partially inducible through a receptor-mediated mechanism, involving different nuclear receptors such as pregnane X receptor, the constitutive androstane receptor, and the aryl hydrocarbon receptor (AhR). Several studies have suggested that AFB1 activates these nuclear receptors, although this has not been firmly established (Arenas-Huertero et al., 2019; Ayed-Boussema et al., 2012; Mary et al., 2015). A recent study revealed that VerA, but not AFB1, can alter the intestinal cell line Caco-2 transcriptome inducing a significant overexpression of several gene targets of the AhR (Gauthier et al., 2020). Among these genes were CYP1A1, CYP1A2, CYP1B1, UGT1A6, IL17F and IDO1. The expression of these genes was not affected by the exposure to AFB1. The same study showed that VerA, unlike AFB1, induces cyto- and genotoxic effects independent of p53 activity. These results suggested that the mechanism of toxicity of both toxins is not the same and that the VerA-mediated activation of AhR might be an important part of its toxicity. Besides, shedding a light on a potential role of the AhR in the toxicological mode-of-action of AFB1 and VerA it may also provide a better understanding of their genotoxic effects and in addition may help to further understand other adverse effects.

In the present study, we aimed at investigating the individual cyto-and genotoxic effects of VerA in liver cells, determine if VerA was able to activate AhR as well as understanding the consequences of such activation when cells are exposed to VerA and AFB1 simultaneously. The HepG2 hepatocarcinoma cell line was chosen as a model for its human and organ relevance, the fact that it expresses CYP450s related to the bioactivation of AFB1 and its wild-type p53 tumour suppressor protein status (Boehme et al., 2010; Westerink et al., 2010; Westerink and Schoonen, 2007).

2. Material and methods

2.1. Chemicals

AFB1 and actinomycin D were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) was purchased from Wellington laboratories (Guelph, Canada). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Valkenswaard, The Netherlands). Analytical grade chloroform, acetic acid and acetonitrile were purchased from Fischer Scientific (Illkirch, France) Stock solutions of the toxins were prepared in DMSO and stored at -20°C until used.

2.2. Synthesis and purification of versicolorin A

VerA was obtained from wheat which was fermented by a pathway-blocked strain of *Aspergillus parasiticus* that specifically accumulates VerA (*A. parasiticus* SRRC 0164). VerA was extracted from wheat using chloroform and purified using an HPLC system following an in-house protocol described elsewhere (Gauthier et al., 2020; Theumer et al., 2018). Briefly, after pre-culture in plates containing malt extract agar at 28°C for 7 days, inoculums were transferred and dispersed in autoclaved wheat and incubated at 28 °C for 7 days. Then, VerA was extracted from wheat and mycelium using chloroform, and the extract was filtered, clarified and evaporated to dryness as previously described (Theumer et al., 2018). The VerA purification was performed with an Ultimate 3000 HPLC

system (Dionex/ThermoScientific, Courtaboeuf, France) using a C18-2 semi-preparative column (7.8 mm id × 25 cm, 5 µm resin, Interchim, Montluçon, France) equilibrated in 0.1% acetic acid (solvent A) and acetonitrile (solvent B). VerA was eluted using 47% solvent B for 17 min and a gradient of 47 to 50% solvent B for 14 min at 4.2 mL/min. The column was rinsed with 90% solvent B for 4 min, and the elution gradient returned to its initial value in 10 min and maintained constant until the end of the run (15 min). After identification using an internal standard (pure VerA), the multiple fractions containing VerA were collected with an ultimate 3000 Fraction Collector (Dionex/ThermoScientific, Courtaboeuf, France) and pooled. Then the solvents were evaporated at low pressure with a Rotavapor® R-215 (Büchi, Flawil, Switzerland). Before toxicity experiments, the identity and purity of the VerA obtained were verified by several methods described previously (Theumer et al., 2018). Stock solution aliquots of VerA were prepared at 10mM using DMSO (Sigma, St Quentin Fallavier, France) and kept at -20°C until used.

2.3. Cell lines and culturing

The HepG2 human liver hepatocellular carcinoma cell line was obtained from the American Type Culture Collection (Manassas, Virginia, United-States). The Dioxin Responsive$_{human}$ Chemical Activated LUciferase gene eXpression (DR$_{human}$ CALUX) cell line consists of HepG2 cells stably transfected with an AhR-responsive reporter and a geneticin resistance plasmid (Budin et al., 2021). The DR CALUX cell line consists of rat H4IIE-cells stably transfected with an AhR-responsive reporter and geneticin resistance plasmid (Murk et al., 1996). The U2OS-based p53 CALUX cell line consists of U2OS cells stably transfected with a p53-responsive reporter construct and geneticin resistance plasmid line (van der Linden et al., 2014). The HepG2-based p53 CALUX cell line (this study) was obtained after the stable transfection of wild-type HepG2 cells with a p53-responsive construct (12xp53RE) described by van der Linden et al. (2014) insulated with sequences of the chicken hypersensitive site-4 gene (Arumugam et al., 2009) and the geneticin resistance plasmid pSG5-neo described elsewhere (Sonneveld et al., 1998).

Wild-type HepG2, DR$_{human}$ CALUX, HepG2-p53 CALUX and U2OS-p53 CALUX cells were maintained in DMEM:F-12 (Gibco) medium supplemented with 7.5% FCS, 10% non-essential amino acids (NEAA), respectively, and streptomycin (10mg/mL) plus penicillin (10U/mL) antibiotics (referred to as maintenance medium). DR CALUX cells were maintained in α-MEM (Gibco) medium supplemented with 10% FCS and streptomycin (10µg/mL) plus penicillin (10U/mL) antibiotics. The assay medium used for exposure to toxins in the CALUX assays consisted of phenol-free DMEM:F12 (Gibco) medium supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS), 10% NEAA and streptomycin (10mg/mL) plus penicillin (10U/mL) antibiotics. Cultures were maintained under standardized conditions in a humidified atmosphere with 5% CO_2 at 37°C. To reduce background AhR activity in DR and DR$_{human}$ CALUX cells exposed for 4 hours, conditioned medium was used as assay medium and consists of sterile-filtered assay medium used to culture DR or DR$_{human}$ CALUX cells (accordingly) for 48 hours (Pieterse et al., 2013).

2.4. Cytotoxicity determination

Cell viability was measured using a luminescence-based assay (CellTiter-Glo, Promega, United-States) as already described (Le et al., 2018). The CellTiter-Glo assay is based on the determination of ATP-levels as an indicator of cell viability and metabolic activity. The assay was performed according to the manufacturer's instructions. Briefly, sub-confluent cells seeded in a 96-well plate were exposed to increasing concentrations of toxins (from 0.1µM to 10µM in log increments) during 24 hours after which reagent were added before luminescence measurement. Concentrations inducing cytotoxicity greater than 20% on average were not considered.

2.5. Gene expression analysis

Sub-confluent wild-type HepG2 cells (70%-95% confluence) seeded in 6-well plates were exposed to 1uM AFB1, VerA or solvent control (DMSO) in assay medium for 24h. Cells were then washed twice in PBS and total RNA was extracted using Extract-all reagent (Eurobio, Les Ulis, France). ARN isolation and RT-qPCR were performed as described elsewhere (Alassane-Kpembi et al., 2017a; Pierron et al., 2016). Data analysis was carried out using LinRegPCR freeware (Ramakers et al., 2003), and normalized against the reference gene Hypoxanthine guanine phosphoribosyltransferase 1 (HPRT1). Primers are presented in Table 1.

Table 1. *primer sequences for SYBR Green quantitative PCR analysis.*

Gene	Accession number	Size (bp)	Sequence
hCYP1A1	NM_000499	102	F – GGTGTTAAGTGAGAAGGTGATTATC R – AGCAGGATAGCCAGGAAGAG
hCYP1A2	NM_000761.5	134	F – GGACTTCTTCCCCATCCTTCG R – GGACACTGTTCTTGTCAAAGTCC
hCYP3A4	ENST00000651514.1	95	F - GGATCCATTCTTTCTCTCAATAA R - AATTTGTAACTTCTCTTGGAAAC
hHPRT1	NM_000194	59	F – AGTAATTGGTGGAGATGATCTCTCAA R – TGACCAAGGAAAGCAAAGTCTG

2.6. CALUX analyses

CALUX reporter cells from continuous culture were resuspended in assay medium to a density of $3x10^5$ cells/mL (DR CALUX) or $2x10^5$ cells/mL (DR_{human} and HepG2-p53 CALUX) or $1x10^5$ cells/mL (U2OS-p53 CALUX). Ninety-six-well plates were seeded with 100uL of the cell suspension per well and incubated under standard conditions (5% CO_2, 37°C) for 24h to reach sub-confluence. Then, cells were exposed in triplicates to a 9-step dilution series in log unit increments of each toxin prepared in assay medium or conditioned medium for 4 hours exposure in DR and DR_{human} CALUX. On each plate, a dilution series of the reference compound (2,3,7,8-TCDD for DR and DR_{human} CALUX and actinomycin D for HepG2-p53 CALUX and U2OS-p53 CALUX) was included. Vehicle

(DMSO) never exceeded 1% in the final dilution. After 4h (DR and DR_{human} CALUX) or 24 hours of exposure (DR, DR_{human} and HepG2 and U2OS p53 CALUX assays), cells were lysed using 5 min shaking in a Triton X-100 buffer. The luciferase activity was measured after the addition of a luciferin-containing solution using a luminometer plate reader (Berthold, Bad Wildbad, Germany).

The software GraphPad Prism (San Diego, California USA) was used for dose-response modelling of DR_{human} CALUX receptor-mediated assay and employs a four parameter nonlinear regression model (Y=Bottom + (Top-Bottom)/(1+10^((LogEC$_{50}$-X)*Hill Slope). Results are expressed as % percentage of maximal induction of the reference compound 2,3,7,8-TCDD (100% corresponding to full receptor activation). Assessed compounds were considered "positive" in the DR_{human} and DR CALUX bioassays when the response of at least one concentration was above the determined threshold of 5% 2,3,7,8-TCDD-equivalent activity with a standard deviation (SD) lower than 20%. For HepG2-p53 and U2OS-p53 CALUX bioassays, the fold-induction per well was calculated by dividing the average Relative Light Units (RLU) level of the tested compound by the average RLU of the solvent control DMSO. In both p53 CALUX bioassays, Actinomycin-D was used as a positive control and tested compounds were considered "positive" when the response of at least one concentration was above the determined 1.5-fold induction threshold and SD<20% (van der Linden et al., 2014).

2.7. γH2AX in cell-western

The γH2AX in-cell western bioassay was performed as previously described (Payros et al., 2017). Briefly, $3.2x10^4$ wild-type HepG2 cells were seeded per well in 96-well plates using assay medium. After 16 hours of incubation under standardized conditions, cells were exposed to toxins for 24 h (in triplicates), fixed with 4% paraformaldehyde (Electron Microscopy Science, Pelanne Instruments, France) in PBS and permeabilized with 0.2% Triton X-100. After blocking (MAXblock Blocking Medium supplemented phosphatase inhibitor PHOSSTOP and 0.1 g. L-1 RNAse A), cells were incubated for 2h with rabbit monoclonal anti-γH2AX (Clone 20E3, Cell signalling) primary antibody. Detection was carried out using infrared fluorescent dye conjugated to goat anti-rabbit antibody (CF770, Biotium), and RedDot2 was added for DNA quantification (Biotium). After 1h of incubation, fluorescence was measured using an Odyssey Infrared Imaging Scanner (Li-CorScienceTec, Les Ulis, France). γH2AX and RedDot2 signals from treated wells were expressed as fold change compared with negative controls. RedDot2 signal was used as a measure of in-well cell viability and expressed as relative cell count (RCC or final cell count ((treated)/final cell count (control) × 100) assessed by automated fluorescence. γH2AX was expressed in fold-change induction of each concentration of toxin relative to vehicle control, in those exposure conditions where cell viability was higher than 50%.

2.8. Data handling

Statistical analyses were performed using GraphPad Prism built-in functions. Differences between groups deemed to be significant with a p-value ≤ 0.05 (*); ≤ 0.01 (**); ≤ 0.001 (***).

3. Results and discussion

3.1. Aflatoxin B1 and versicolorin A show similar cytotoxicity in HepG2 cells

The time and dose-dependent cytotoxic effects of AFB1 and VerA were determined to define non-cytotoxic exposure conditions for subsequent experiments. HepG2 cells were exposed to increasing concentrations of AFB1 or VerA (0.1, 0.3, 1, 3, 10 and 30µM) during 24h or 48h and the effect on cell viability was determined using the CellTiter-Glo® assay. Results presented in Figure 1 show that, for both AFB1 and VerA, the percentage of viable cells at 48 hours decreased at concentrations higher than 1µM in a dose-response manner. After 24 hours of incubation, a slight effect was visible but no concentration resulted in a decrease in viability higher than the threshold of 20%. The maximal cytotoxicity after 48h was obtained at 10µM for both toxins. For subsequent experiments, the incubation time was set at 24 hours. The cytotoxicity we report for VerA after 48h of incubation is in line with have been reported by Gauthier et al (2020) in HCT116 and Caco-2 cell lines using the same method. At 24h of incubation, Theumer et al. (2018) reported higher cytotoxicity for VerA in HepG2 cells using a different method (fluorescence-based). Overall, our results show that in HepG2 cells, VerA and AFB1 have similar cytotoxic potency which results in significant cytotoxic effects after 48h of incubation.

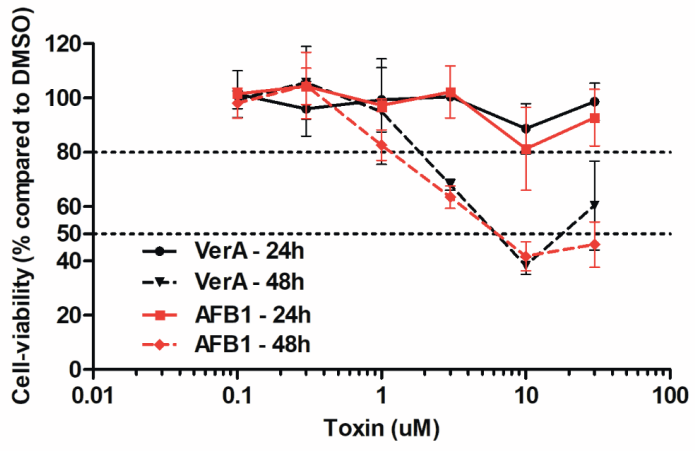

Figure 1. *Effects of versicolorin A (VerA) and aflatoxin B1 (AFB1) on cell viability in HepG2 cells measured using the CellTiter-Glo® assay (mean ± SD)*

3.2. Comparative genotoxicity of versicolorin A and aflatoxin B1

We subsequently compared the genotoxicity of VerA and AFB1 in HepG2 cells through the evaluation of the induction of H2AX phosphorylation, Serine15-p53 phosphorylation and p53 tumour repressor induction, and compared these results with p53 tumour repressor induction in cells devoid from phase I enzymes (U2OS cells).

We observed that in HepG2 cells both AFB1 and VerA elicited a dose-dependent increase of γH2AX levels (Figure 2A) and p53 transcriptional activity (Figure 2B). HepG2 cells exposed to 1uM of each toxin for 24h also showed a higher abundance of phosphorylated-p53 (Ser15) (Figure 2C). AFB1 was a more potent inducer of the phosphorylation of H2AX and p53 than VerA, whereas the p53 transcriptional response induced by both toxins was similar. In the case of γH2AX induction, the lowest tested concentration at which levels significantly induced genotoxicity, referred as lowest observed effect concentration (LOEC; first concentration >1.2-fold induction in the γH2AX-ICW bioassay), were a factor of 33 different, equal to 0.3µM and 10µM for AFB1 and VerA respectively.

The LOEC values we report for AFB1 are in the range of those obtained by others in γH2AX-ICW bioassays performed in HepG2 cells (Khoury et al., 2013; Theumer et al., 2018). In the HepG2-p53 CALUX bioassay, the LOEC (>1.5-fold induction in the HepG2-p53 CALUX bioassay) was equal to 1µM for both AFB1 and VerA. In the case of AFB1, our reported LOEC value is in line with those obtained with another HepG2-based p53 reporter gene assays (Westerink et al., 2010) and an ELISA-based assay (Boehme et al., 2010). Regarding induction of p53 Ser15 phosphorylation by AFB1 and VerA, our results are in line with previous reports in intestinal HTC116 cells (Gauthier et al. 2020). Additional experiments using the p53 CALUX U2OS cells, a cell line that does not show appreciable levels of CYP450 activity (van der Linden et al., 2014; van Vugt-Lussenburg et al., 2018), showed that neither AFB1 nor VerA increased p53 transcriptional activity (Supplementary Figure 2). This indicates that the metabolic status of the exposed cells defines the presence and magnitude of p53 induction for both toxins. Our results suggest that both AFB1 and VerA are genotoxic to human liver cells following cellular events dependent on the metabolic status of the cell. However, the molecular mechanisms leading to these genotoxic effects seem to differ, as AFB1 induced greater phosphorylation of the key proteins H2AX and p53 while both toxins induced similar levels of p53 transcriptional activity.

The genotoxic mechanism of AFB1 is well known. The bioactivation of AFB1 by CYP450s results in the production of AFBO, a metabolite that spontaneously and irreversibly attaches to guanine residues to generate highly mutagenic DNA adducts. These adducts lead to the promotion of a mutational effect at the codon 249 in exon 7 of the p53 tumour suppressor gene. Ultimately, this mutation provokes the development of hepatocellular carcinoma which is a major adverse outcome related to aflatoxin exposure (Hamid et al., 2013). Contrastingly, little is known regarding the molecular mechanisms leading to the genotoxicity of VerA. Previous reports on VerA and AFB1 toxicity suggested a different mode of action for each mycotoxin (Gauthier et al., 2020; Theumer et al., 2018). The study

of Gauthier et al. (2020) also revealed a differential transcriptomic response in intestinal Caco-2 cells exposed to these toxins, with VerA being a significantly stronger disruptor of gene expression, as well as being more genotoxic. Furthermore, VerA genotoxicity was observed in cells with limited bioactivation capacity, suggesting that VerA may be genotoxic as a parent compound independent of its bioactivation by CYP450s (Gauthier et al., 2020; Theumer et al., 2018). Nonetheless, the formation of DNA-reactive species during VerA metabolism by CYP450 cytochromes is structurally possible (reactive double-bond, Supplementary Figure 1), but has not been confirmed yet.

Besides the characteristic AFB1-like reactive double-bond, other structural features of VerA (anthraquinone with β-hydroxy, Supplementary Figure 1) suggest that it would be able to induce mitochondrial respiratory chain uncoupling associated with subsequent induction of severe oxidative stress (Gauthier et al., 2020; Theumer et al., 2018). Therefore, it is likely that reactive oxygen species (ROS) accumulating during this process can induce DNA damage. Besides, the ability of VerA to induce higher oxidative stress than AFB1 was demonstrated in intestinal Caco-2 cells (Gauthier et al., 2020). Two main pathways, namely the ATM-Chk2 axis and the ATR-Chk1 axis govern p53 and H2AX phosphorylation and subsequent DNA damage-mediated cell cycle arrest. Of these, the ATM-Chk2 axis responds to DNA double-strand breaks (DSBs) whereas the ATR-Chk1 axis responds to replication stress (RS; Shaltiel et al., 2015). The p53 protein can be phosphorylated at different sites depending on the state of activation. The phosphorylation of the Ser15 is known to be mediated by Chk2 and is an early event that is specific of DSBs-dependent p53 activation. Also, the level of phosphorylation of p53 at Ser15 is known to correlate with the degree of genotoxic stress induced by DSBs (Ichwan and Ikeda, 2008). Gauthier et al. (2020) has shown that during the genotoxic stress induced by AFB1 and VerA, ATR is activated before ATM, suggesting that DNA DSBs occur after RSs. However, VerA was able to induce an earlier and greater RS than AFB1, which in turn might be the trigger to DSB formation. Our results support these findings and suggest a similar mode of action in the liver emphasizing that despite sharing structural features, both toxins show some differences in their genotoxicity mechanisms. While AFB1 genotoxic effects are attributed to a bioactivated metabolite VerA genotoxic effects may be attributed to both a bioactivated metabolite (Supplementary Figure 1, green dashed circle) and the induction of oxidative stress through its β-hydroxylated anthraquinone structure (Supplementary Figure 1, blue dashed circle).

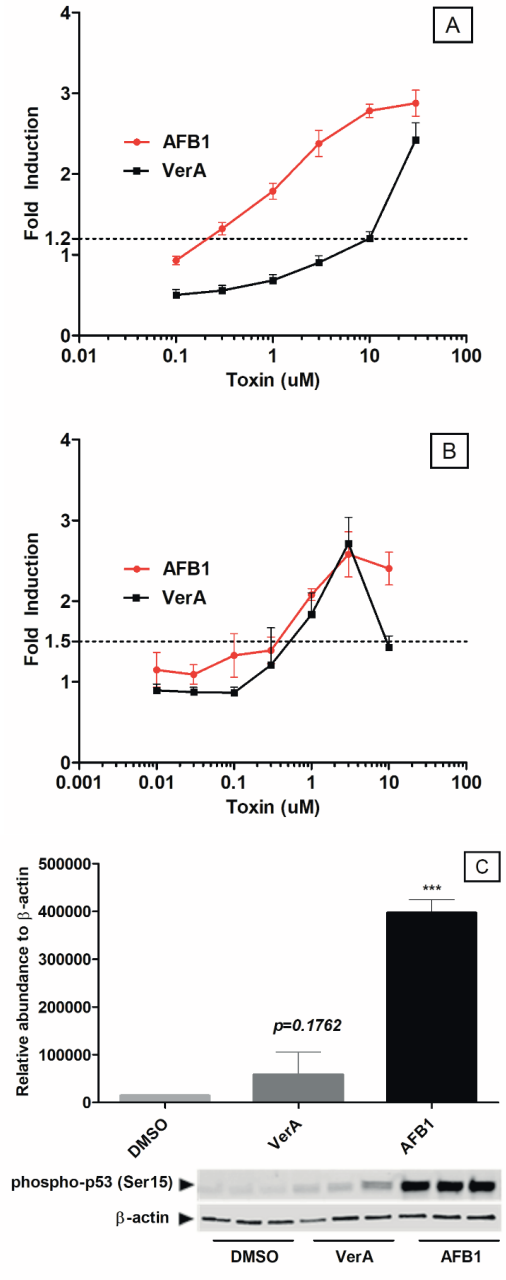

Figure 2. *Individual effects of aflatoxin B1 (AFB1) and versicolorin A (VerA) on (A) H2AX phosphorylation in HepG2 cells measured using the yH2Ax-ICW bioassay, on (B) p53 transcriptional activity in the HepG2-p53 CALUX bioassay and on (C) on the p53-Ser15 phosphorylation in wild-type HepG2 cells exposed to 1μM of each toxin, or vehicle control (DMSO) for 24h (mean ± SD).*

3.3. Comparative Induction of AhR and expression of CYP450 enzymes by versicolorin A and aflatoxin B1

To study the effect of both toxins on AhR activation, AhR-responsive DR_{human} CALUX reporter cells were exposed to non-cytotoxic concentrations of AFB1 and VerA. As shown in Figure 3, we did not observe a significant dose-dependent increase of AhR transactivational activity for AFB1 (1.3% of 2,3,7,8-TCDD equivalent activity) after 4 hours of incubation. By contrast, VerA significantly induced AhR-transactivation in a dose-dependent manner up to 52% of the 2,3,7,8-TCDD equivalent activity with a calculated EC_{50} of 0.9µM and calculated LOEC of 0.2µM (>5% 2,3,7,8-TCDD equivalent activity in the DR_{human} CALUX bioassay). When toxins were incubated for 24 hours, a strong decrease in AhR-mediated luciferase induction by VerA was observed (Supplementary Figure 3A), thus supporting our earlier finding that cellular metabolism negatively influences the concentration of VerA in the cell. The effects of AFB1 and VerA on AhR transactivation were confirmed in another (metabolically competent) AhR-responsive cell line, the DR CALUX, in which we obtained similar results for both toxins (Supplementary Figure 3B).

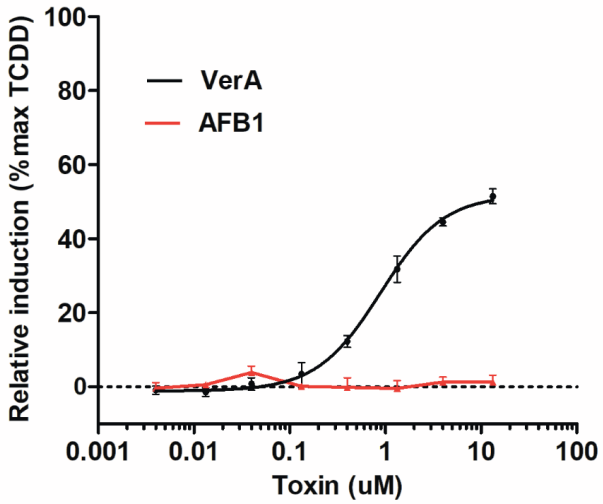

Figure 3. *Effects of versicolorin A (VerA) and aflatoxin B1 (AFB1) on the AhR-mediated luciferase induction in HepG2 DR_{human} CALUX reporter cells after 4 h of incubation (mean ± SD).*

The AhR is a latent cytoplasmic transcription factor involved in xenobiotic sensing, induction of CYP1A1/2 as well as other cellular processes such as cancer progression and control of the cell-cycle (Dietrich and Kaina, 2010; Esser and Rannug, 2015; Larigot et al., 2018). A previous transcriptomic analysis on intestinal Caco-2 cells revealed significant up-regulation of AhR target genes in cells exposed to VerA, including several CYP450 enzymes, whereas AFB1 did not induce such changes (Gauthier et al., 2020). These results suggested an involvement of AhR signalling in VerA toxicity, which has not been

investigated yet. Moreover, typical ligands of the AhR, such as dioxins and certain dietary compounds, display a planar chemical structure (Denison and Nagy, 2003), which further suggested that VerA could interact with the receptor and affect normal cell physiology through activation and/or disruption of normal AhR signalling. The present results indicate that VerA can activate the Ah-receptor which is consistent with gene expression results found in Caco-2 cells (Gauthier et al., 2020).

Then, we determined if VerA-dependent activation of the AhR was associated with the induction of CYP450 enzymes expressed in HepG2. That is CYP1A1/2 and CYP3A4 (Boehme et al., 2010; Westerink and Schoonen, 2007). The results, presented in Figure 4, indicate a pattern of induction where VerA significantly increased the expression of CYP1A1 (2,4-fold) mRNA whereas AFB1 significantly increased the expression of CYP1A1 (2,2-fold) and CYP3A4 (1,5-fold). In the case of AFB1, results are in line with reported induction of CYP3A4 and CYP1A1 transcription by AFB1 in different cell types (Ayed-Boussema et al., 2012; Boehme et al., 2010). There was no significant induction of CYP1A2 for both toxins. For VerA, our results in a human liver cell line are in accordance with those reported by Gauthier and co-workers in human Caco-2 intestinal cells although induction of CYP1A1 by VerA in HepG2 cells was lower (Gauthier et al., 2020). Our results show that VerA can induce CYP1A1 mRNA transcription, opening the question that the toxin may be a possible substrate of this CYP450 isoform, as suggested by others (Gauthier et al., 2020) and our earlier results regarding genotoxicity. In our study, the elevation of CYP1A1 mRNA level was also associated with significantly increased AhR transactivational activity. This suggests an AhR-mediated mechanism for CYP1A1 induction by VerA and/or an effect on AhR signalling and a possible role of the AhR in the genotoxicity of VerA.

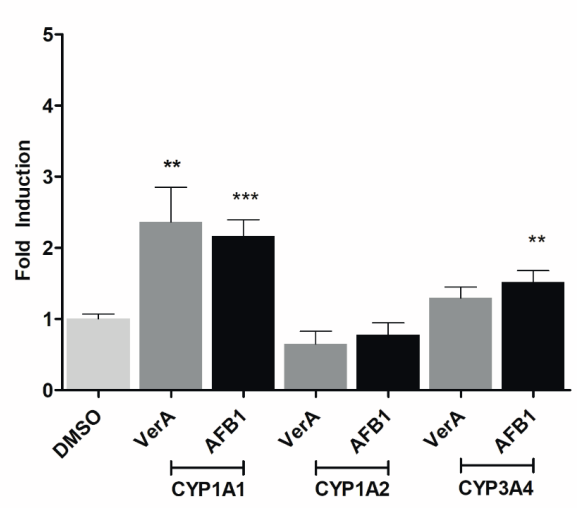

Figure 4. *Effects of 1µM versicolorin A (VerA) and 1µM of aflatoxin B1 (AFB1) on the mRNA transcription induction of CYP1A1/2 and CYP3A4 in HepG2 cells (mean ± SD). *: p-value ≤ 0.05; **: ≤ 0.01; ***: ≤ 0.001.*

The VerA-mediated induction of the AhR suggests a potential enhancing effect of AFB1 genotoxicity as the ligand-binding activation of the AhR results in increased expression of CYP450s that can catalyse the bioactivation of procarcinogens. This mechanism has been well described in the case of polyaromatic hydrocarbons, such as benzo[a]pyrene, whose AhR-mediated bioactivation by CYP1A1 results in the production of DNA-reactive PAH metabolite forming mutagenic DNA adduct and DSBs (Smit et al., 2017). Therefore, when AFB1 and VerA are present simultaneously, the AhR-mediated CYP1A1 inducing properties of VerA may support the formation of AFB1 reactive metabolites and enhance related DNA damages.

Furthermore, the interaction of VerA with AhR signalling indicates a possible role of the AhR in the AFB1-independent genotoxicity mechanisms of VerA itself. Regarding the hypothesis that certain aspects of VerA genotoxicity may be attributed to DNA damages resulting from oxidative stress, it should be noted that the AhR plays a significant role in the upstream events of p53-mediated cell-cycle progression/apoptosis. This typically is exerted through the control and regulation of ROS levels via the induction and repression of certain CYP450s and non-CYP450 enzymes such as UGT1A6 (Nebert et al., 2000). Thus, the alteration of normal AhR signalling by VerA may result in a perturbation of that balance and lead to ROS formation and subsequent elevated oxidative stress. Considering the importance of the AhR in normal and disturbed physiology, in several organs such as the liver or the intestine (Larigot et al., 2018; Safe et al., 2020, 2013) future investigations should aim at further elucidating the relationship of VerA with AhR events related to cell-cycle which may help to better understand the toxicity mechanisms of VerA.

3.4. Versicolorin A enhances the genotoxicity of aflatoxin B1

As VerA and AFB1 may co-occur in food, we next studied the effect on cytotoxicity and genotoxicity of VerA in combination with AFB1, using increasing concentrations of AFB1 with 1µM of VerA. This concentration of VerA induced a significant activation of AhR and CYP1A1 expression over a 24h time-period of exposure but did not have a significant cytotoxic effect nor induced a significant increase of γH2AX or phospho-p53. Results showed that exposure to this mixture resulted in a stronger cytotoxic effect compared to that of 1uM VerA alone, as well as the effect of AFB1 alone, in particular after 48h of exposure (Figure 5). Exposure of HepG2 cells to the mixture resulted in significantly higher levels of yH2AX induction than the effect of 1uM of VerA alone and the effect of AFB1 alone from 0.1µM to 3µM (Figure 6A). The same effect was observed in the HepG2-p53 CALUX where the combination resulted in significantly greater levels of p53 transcriptional activity than 1µM of VerA alone and 0.1µM to 1µM of AFB1 alone (Figure 6B), as well as regarding Ser15-p53 phosphorylation at 1µM (Figure 6C). Interestingly, genotoxicity was enhanced especially in those mixtures containing relatively low AFB1 concentrations.

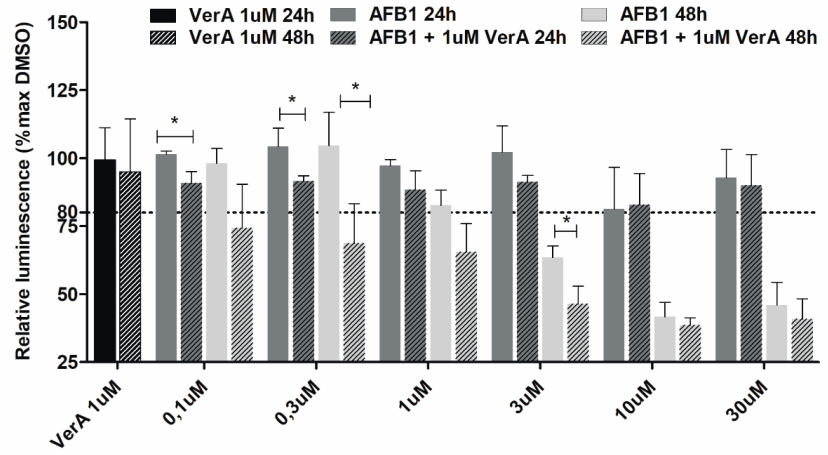

Figure 5. *Effects of versicolorin A (VerA) and aflatoxin B1 (AFB1) and mixtures of AFB1 + 1uM of VerA on cell viability in HepG2 cells measured using the CellTiter-Glo® assay (mean ± SD). *: p-value ≤ 0,05.*

The finding that low concentrations of AFB1 in combination with 1µM of VerA elicit significant cyto- and genotoxic effects is highly relevant to food hazard assessment. Other studies have highlighted the effects elicited by the combination of AFB1 with other regulated mycotoxins such as fumonisin B1, ochratoxin B1 and deoxynivalenol (Alassane-Kpembi et al., 2017b; Corcuera et al., 2011; Mary et al., 2015; McKean et al., 2006). Combined effects of AFB1 with fumonisin B1, deoxynivalenol and T-2 toxin on endpoints such as cell viability, cytotoxicity indicated additivity and synergism, although the magnitude may vary depending on the cell system. Combination of AFB1 and carcinogenic mycotoxins, ochratoxin A and fumonisin B1, also suggest enhanced genotoxicity via mechanisms which remain to be understood. Our results are in line with these, but also emphasize that interactions can also occur between toxins from the same family and that more frequently than not, toxicity from mixtures cannot be explained by the toxicity of individual toxins. This is relevant for regulatory issues since regulations regarding mycotoxins levels in food and crops generally focus on the identification and quantification of a handful of mycotoxins, which are often final fungal metabolites. However, the occurrence and concentrations of numerous biosynthetic precursors of mycotoxins that are normally overlooked are recently being communicated, revealing their importance (Abdallah et al., 2017; Janić Hajnal et al., 2020; Kovalsky et al., 2016). Mycotoxins are always found as complex mixtures rather than as single compounds in foodstuffs, and there is a need in expanding the toxicological knowledge regarding the toxicity of biosynthetic precursors, and the toxic effect of complex mycotoxin mixtures (Streit et al., 2013). AFB1 and VerA show a similar structure, and it was anticipated that the toxicity profile and mode of action may be similar and thus elicit combined effects (Speijers and Speijers, 2004). However, here we described that VerA and AFB1 do not share the same mechanism of genotoxicity nor the same kinetics in the activation of DNA damage responses. Indeed, our results indicate that VerA increases AFB1 toxicity by

promoting its bioactivation and promoting additional genotoxic stress. These new aspects of the toxicity of VerA, different from those of AFB1, that could explain that the combined exposure to AFB1 and VerA lead to more deleterious effects than AFB1 alone. Future research should be conducted to fully characterise the interaction of VerA and AFB1 and the nature of their interaction at all concentrations. Moreover, it will be important to verify if the AhR activation promoted by VerA is associated with a greater bioactivation of AFB1 or if the interaction relies in their apparent different genotoxicity mechanisms.

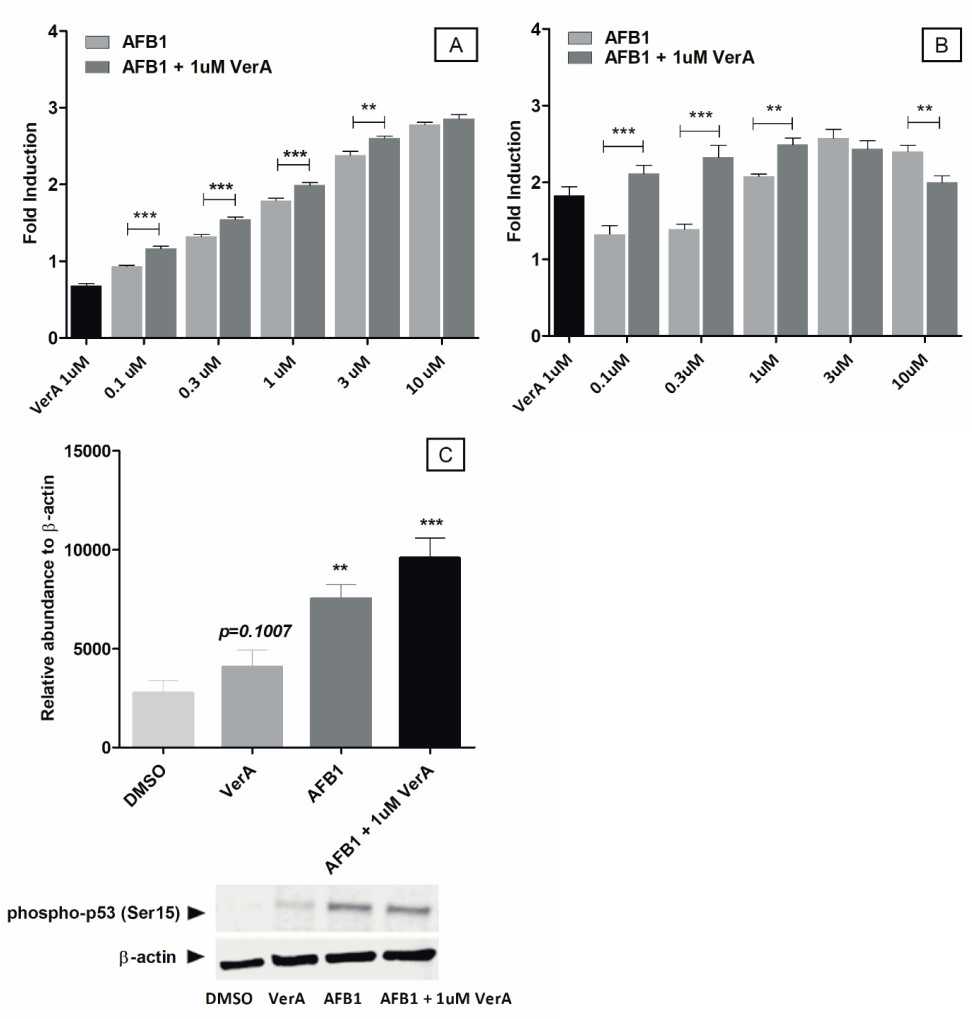

Figure 6. *Combined effects of aflatoxin B1 (AFB1) and 1uM of versicolorin A (VerA) on (A) H2AX phosphorylation in HepG2 cells measured using the yH2Ax-ICW bioassay and (B) p53 transcriptional activity in HepG2-p53 CALUX bioassay and (C) p53-Ser15 phosphorylation in wild-type HepG2 cells compared to their individual effects (mean ± SD). **: p-value ≤ 0.01; ***: ≤ 0.001.*

4. Conclusion

The toxicological knowledge regarding the effects of AFB1's precursors is scarce at best and, to our knowledge, there have not been studies evaluating the combined genotoxic effect of AFB1 and its precursors in human cells. In this study, we applied *in vitro* bioassays and methods to evaluate and compare the toxic effects of VerA and AFB1. The cytotoxicity and genotoxicity of VerA in HepG2 cells were reflected by the decrease of cell viability and the induction of H2AX phosphorylation, serine15-p53 phosphorylation and p53 tumour repressor transcriptional activity. Differential genotoxic responses suggested differences in mechanisms of action of VerA and AFB1 unlike AFB1, the main genotoxic mechanism of VerA does not seem to involve the formation of DSBs by VerA-epoxide DNA reactive metabolites. VerA was able to induce CYP1A1 transcription via an AhR-mediated mechanism which suggested that VerA can influence AFB1 toxicity by promoting its bioactivation by CYP450s. The addition of 1µM of VerA to increasing concentrations of AFB1 resulted in significantly enhanced AFB1 cyto- and genotoxic effects thus, supporting the hypothesis that the AhR activation promoted by VerA could be associated with a greater bioactivation of AFB1. The present results pinpoint to a need of expanding the toxicological knowledge regarding mycotoxin biosynthetic precursors with the aim of identifying those who may pose a threat to food safety. Since the exposure to multiple mycotoxins is a rule rather than an exception, our results additionally contribute valuable information to hazard identification and risk assessment in food safety.

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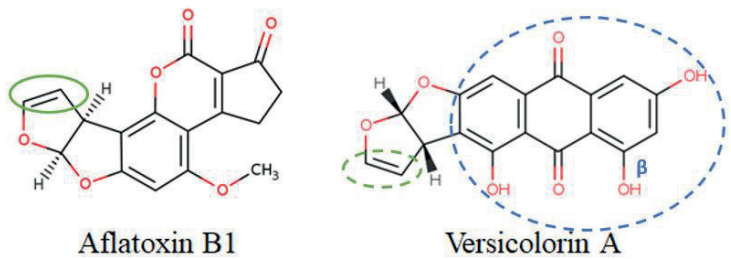
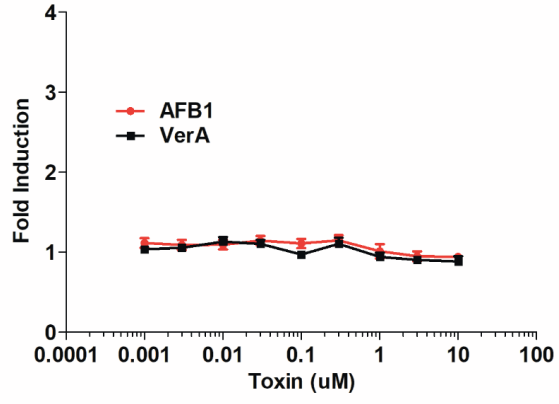
Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

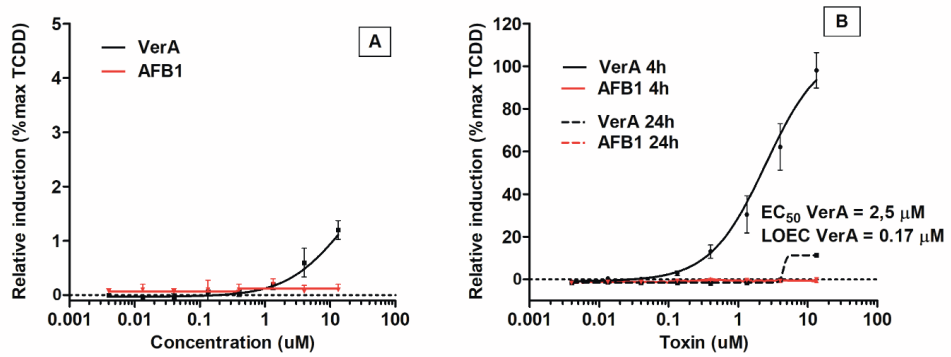
Supplementary data



Supplementary Figure 1. *Chemical structure of aflatoxin B1 (AFB1) and versicolorin A (VerA). In the AFB1 structure, the green circle highlights the known double bond target of CYP450s enzymes. In the VerA structure, the green circle indicates the putative reactive double bond, and the dashed blue circle highlights the anthraquinone and its β-hydroxy group.*



Supplementary Figure 2. *Individual effects of aflatoxin B1 (AFB1) and versicolorin A (VerA) on p53 transcriptional activity in the U2OS-p53 CALUX bioassay (mean ± SD).*



Supplementary Figure 3. *Effects of versicolorin A (VerA) and aflatoxin B1 (AFB1) on the AhR-mediated luciferase induction in (A) DR_{human} CALUX cells after 24 hours of incubation and in (B) DR CALUX reporter cells after 4-24 hours of incubation (mean ± SD).*

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Chapter 6

General discussion & future outlook

The studies presented in this thesis resulted in the development of improved aryl hydrocarbon receptor (AhR) based CALUX bioassays that aim to enhance human relevance and provide better interpretability of bioassay results towards human exposure. The existence of species-specific differences in AhR specificity and sensitivity was considered because of its potential implications for human-health based hazard assessment. The bioassays were applied in studies with public health implications such as the presence of dioxin-like AhR-contaminants in plastic toys (chapter 3); smoking-related PAH exposure and the potential effect on foetal development (chapter 4) and mycotoxin mixture toxicity (chapter 5). Overall, significant improvements were made with the development of two improved bioassays and we provided some future domains of application for the developed methods. This will help to better comprehend the effect of AhR-active chemical mixtures on human health and identify them in the environment.

1. Improvements of AhR-based bioassays with a focus on human exposure and impact assessment

In this thesis, two novel AhR-mediated bioassays were developed to respond to relevant needs in toxicological risk assessment, which are increased human relevance and comprehensive analysis of AhR-active mixtures.

1.1. The DR_{human} CALUX bioassay

The first bioassay developed (chapter 2) was a human-hepatocellular carcinoma (HepG2) cell-based AhR reporter gene assay, the DR_{human} CALUX. Various AhR-mediated reporter gene assays (e.g., rat H4IIe-based DR CALUX; mouse hepa1c1c7-based CALUX) have been developed in the past, with a focus on high(est)-possible sensitivity to measure minute amounts of dioxins in e.g., food. However, there is at present still an under-representation of standardized AhR-based bioassays with optimal human relevance to improve the predictability and interpretability of the possible impact for public health. Besides, there was no human-based AhR transactivation assay in the CALUX® panel (van der Burg et al., 2013).

The novel DR_{human} CALUX appears to be more sensitive than a majority of the existing HepG2-based AhR-reporter assays based on the EC_{50} for 2,3,7,8-TCDD and LOD for 2,3,7,8-TCDD (Table 1). Information on the stability over passages was only found for the AZ-AhR cell line, which appears to be twice less stable than DR_{human} CALUX cells. Moreover, the DR_{human} CALUX has the additional advantage of being compatible with the miniaturized (384-well plates) CALUX automated high-throughput screening platform, which seems to only be the case for the HG2L7.5c1 that is used in EPA's TOX21 screening programme (1536-well format, https://tripod.nih.gov/tox21). The DR_{human} CALUX bioassay shows high inducibility (75 to 100-fold in 96-well format, chapter 2) which is relevant for identifying weaker ligands of the receptor and bioanalysis after short incubation periods (4 hours in chapter 5) as signal-to-background ratio allows a larger resolution window to identify active chemicals that may not be detectable after 24h of incubation (e.g., because of cell metabolism).

Table 1. *Comparison of the DR_{human} CALUX with existing HepG2-based AhR reporter gene assays.*

Cell line	DREs	$EC_{50\ TCDD}$ (M)	LOD_{TCDD} (M)	Passage	Compatible with HTS	Reference
DR_{human}	4	3.00E-10	6.50E-12	30	yes	Chapter 2
AZ-AhR	4	2.00E-09	NC	15	NC	(Novotna et al., 2011)
TV101	4	1.00E-09	1.00E-10	NC	NC	(Anderson, 1995)
HG2L1.1c3	4	2.50E-10	5.00E-12	NC	NC	(He et al., 2011)
HG2L6.1c1	4	2.20E-09	1.00E-10	NC	NC	(Brennan et al., 2015)
HG2L7.5c1	20	2.20E-10	3.00E-11	NC	yes	(Brennan et al., 2015)

DRE: dioxin responsive element; LOD: limit of detection; HTS: high-throughput screening; NC: not communicated

Regarding the choice of the host cell line, the HepG2 cell line was selected for its organ relevance concerning dioxins' distribution in the human body, known relative sensitivity to AhR ligands, as well as its suitability for use in high-throughput bioassays. The choice to use a similar reporter construct than the one expressed in the DR CALUX makes *in vitro* species differences investigation possible, and likely more accurate. Indeed, the main difference between the two CALUX cell lines is the host cell line. The sensitivity of the DR_{human} CALUX is around 10 to 100-fold lower than the rat-H4IIe based DR CALUX cell line, which is consistent with known species differences and its relevance to impact assessment for human and public health. However, it is less suitable as a bioassay for routine analysis of the very small amounts of dioxins present e.g. in food items. Therefore, the DR_{human} CALUX cell line developed is a valuable addition to the panel of AhR CALUX bioassays, with a higher human relevance, than e.g., the rat H4IIe-based DR CALUX cell line.

There are several future applications for the DR_{human} CALUX. The first one would be the screening of compound libraries using the (HTS) DR_{human} CALUX to identify human-AhR active chemicals and, if necessary, make species comparison with the DR CALUX cell line. Even though it is less suitable than rodent-based cell lines for the regulatory screening of dioxin-like activity in food, it is still relevant to be used in studies where higher levels of AhR-activity are expected (e.g. sediments, plastics), and in the objective of depicting the activity in a human cellular context. In fact, the P450RGS bioassay employing the TV101 cell line (Anderson, 1995; Postlind et al., 1993) is an example of an official test method for the screening of environmental samples which uses a human-based reporter gene assay (US EPA, 2015). Thus, the DR_{human} CALUX, which is more sensitive than the TV101 cell line (Table 1), may be suitable for such an application. Another type of application for the bioassay would be epidemiological studies that focus on endogenous ligands, dietary, and microbial-derived AhR-activity in human samples which appear to require species relevance due to specific differences in ligand specificity (Hubbard et al., 2015; Rothhammer et al., 2018). This would require to first determine if the bioassay is responsive to endogenous ligands, which alike PAHs are often rapidly metabolised.

However, we have seen in chapter 5 that AhR induction can be detected at 4h of exposure in the DR_{human} CALUX, cells which may facilitate the analysis of such compounds.

1.2. $DR_{high-performance}$ CALUX (DR_{hp})

The second bioassay developed in this thesis and described in chapter 4 is a highly sensitive, AhR-based reporter gene assay which was further optimized for PAH determination in small amounts of human tissue. In human epidemiological studies, particularly studies focusing on early (foetal) development, the amount of tissue material available for analysis is always very small and therefore analyses on these small sample volumes may only be feasible when it is possible to apply ultra-sensitive, high-performance bioassays for analysis. Thus, the development of the DR_{hp} CALUX was motivated to respond to that need of developing high-performance AhR-based reporter gene assays for ultrasensitive analysis of low levels of activity in small volumes of samples. The availability of a DR_{hp} CALUX method would enable us to quantify foetal- and early-life levels of exposure to mixtures of AhR-active chemicals such as PAHs and dioxins.

As rodent-derived continuous cell lines are amongst the most sensitive cell lines for which response to prototypical AhR ligands (e.g., dioxins, PAHs) is relatively well described, the H4IIE cell line was selected and enhancements were driven by plasmid construction engineering. As a result, the DR_{hp} CALUX bioassay was constructed to contain 20 dioxin-responsive elements (DREs) integrated within the luciferase reporter construct, stably integrated into the genome of stably transfected H4IIe cells. The aim was to improve the bioassay's limit of detection and luciferase reporter responsiveness, which are important parameters to consider for the quantification of bioactivity equivalents as this means that lower amount of activity can be quantified while retaining a sufficient analytical window for more precise quantification. This DR_{hp} CALUX cell line shows an improved sensitivity (compared to the limit of detection LOD, and EC_{50} of other H4IIE-based AhR CALUX bioassays) particularly towards non-stable PAHs as well as enhanced luciferase responsiveness (Table 2). The cell line can also be operated optimally at 4h of incubation, with a LOD for total PAH analysis of 7 pg B[a]P-equivalent per gram of tissue sample (chapter 4). This enabled the use of very small sample amounts of approximately 200 mg on average of human (foetal) tissue for PAH analysis. To our knowledge, this is the first time that a report on such a sensitive, total PAH-based bioassay is published, which can be used optimally for human early life stage epidemiological and monitoring studies requiring only minute amounts of human tissue.

Table 2: *Comparison of H4IIE-based AhR responsive CALUX bioassays.*

Cell line	DREs	Reference compound	EC_{50} (M)	LOD (M)	Maximal fold induction	Reference
DR_{hp} CALUX	20	2,3,7,8-TCDD	2.4E-12	1.0E-13	57	Chapter 4
		B[a]P	8.9E-12	8.9E-12	151	Chapter 4
DR CALUX	4	2,3,7,8-TCDD	6.5E-12	5.0E-13	14	Chapter 4
PAH CALUX	4	B[a]P	3.0E-09	5.2E-11	288	(Pieterse et al., 2013)

DRE: dioxin responsive element; LOD: limit of detection

Hence, a perspective on future applications of the DR_{hp} CALUX bioassay, based on the study presented in chapter 4 would be the quantification of PAH-mediated activity in minute amounts of foetal samples e.g., cord blood and reproductive tissue, to better understand the impact of PAHs on human development. In addition, the DR_{hp} CALUX would also be relevant to assess foetal-developmental exposure to dioxins and dioxin-like chemicals. Another likely application would be the regulatory screening of dioxins in food since a lower LOD for 2,3,7,8-TCDD than the DR CALUX technically implies that less sample can be used. Consequently, this would reduce the volumes of organic solvents used for extraction, therefore, making the analysis more environmentally friendly.

2. Demonstration of the applicability the novel AhR-based CALUX bioassays in human-oriented hazard assessment of chemicals and mixtures thereof

After developing the two optimized AhR-mediated CALUX bioassays, the next step was to demonstrate their applicability. This was done through studies focusing on human exposure and aiming at assessing AhR-mediated activity elicited by chemicals and mixtures of chemicals with an emphasis on classes of chemicals (dioxins, PAHs, mycotoxins), contexts, and adversities with human health-relevance.

2.1. Performances of the DR_{human} CALUX and DR_{hp} CALUX

Before applying the bioassays in the different studies attention was paid to ensure that the two bioassays were reliable and reproducible for these applications, which is, in fact, required for the further implementation of an *in vitro* method for regulatory use in safety assessment (*"Guidance on good in vitro method practices"* OECD, 2018). This is the reason why the first steps after the development of the DR_{human} and DR_{hp} CALUX cell lines were to pre-validate the bioassays based on their future application in chapter 2 for DR_{human} and chapter 4 for DR_{hp} CALUX. Criteria such as the stability over passages, coefficients of variation ($\%CV_{EC50}$), reproducibility ($\%VC_{R\ EC50}$), limits of detection (LOD) and quantification (LOQ) were determined to ensure reliable analysis. The good performances of both methods indicated their reliability and suitability for the studies performed.

2.2. Hazard assessment of individual chemicals

Currently, most *in vitro* AhR-related toxicity data are generated in cell lines originating from a non-human origin (e.g., rat, mouse, guinea pig) which may represent a weakness with respect to human relevance, particularly given the fact that there are significant differences in species sensitivity for the different classes of AhR ligands (Connor and Aylward, 2006; Denison et al., 2002; Denison and Faber, 2017; Hahn et al., 2017). However, by using *in vitro* human cell-based methods, it is possible to provide human-based *in vitro* data which may significantly enhance the predictability and relevance of suspected effects in humans. Using DR_{human} CALUX we determined human-relevant potencies for a subset of 21 PCDD/Fs and PBDD/Fs (chapter 2). We also identified a new (potent) naturally occurring ligand of the human AhR, the mycotoxin versicolorin A, a precursor of aflatoxin B1 (chapter 5), with potentially significant implications for mycotoxin-related genotoxicity, which warrants to be further investigated.

Comparisons were also made between the human HepG2-based DR_{human} CALUX and the rodent-based DR CALUX to elaborate on the similarities and species-differences regarding AhR-signalling in terms of hazard prediction. Similarities between species in the two studies (chapter 2 and 5) were that the PCDD/Fs, PBDD/Fs congeners active in the DR_{human} were also found to be active in the rodent-based DR CALUX. In addition, similar time-dependent induction, e.g., active at 4h and almost inactive at 24h of exposure, of AhR transactivation was observed for versicolorin A in both bioassays while aflatoxin B1 was inactive in both bioassays at both time points. Species differences in sensitivity were, however, observed, e.g., in the case of PCDD/Fs and PBDD/Fs. A typical, 10 to 100-fold sensitivity difference based on the EC_{50} was observed between cell lines, with the rodent AhR-based reporter cell line being more sensitive than the human-based AhR-responsive cell line. This is in line with the notion that humans are (much) less sensitive to dioxin-like toxicity than rodents (Birnbaum, 1994). Overall, both human and rodent AhR-reporter cell lines have been shown to be able to reliably estimate the relative toxic potency of different chlorinated and brominated dioxins and furans. Besides, they showed good predictability in comparison to the WHO-based relative potencies for PCDD/Fs and PBDD/Fs.

2.3. Assessment of complex (AhR-active) mixtures

Historically, estimates of total toxicity of complex mixtures of e.g., dioxins are estimated using a toxic-equivalency factor (TEF)-based approach. In this approach the concentrations of 17 PCDD/Fs (or 29 when including dioxin-like PCBs) are quantified by analytical-chemical methods, followed by multiplication of those congeners' concentrations with their toxic equivalency factor (e.g. WHO-TEFs) and adding those up to one total toxic equivalency (TEQ), expressed in e.g., ng TEQ per g matrix. For PAHs, only 16 of the many hundreds of PAH congeners are routinely tested, the EPA-16. There are yet, no consensus TEFs established by the WHO for PAHs. Nevertheless, in a recent study using an AhR-based reporter assay, the PAH CALUX, the relative AhR-inducing potency for individual PAH congeners was determined and these potencies compared

well with their genotoxic/carcinogenic potency (Pieterse et al., 2013) and can thus be used when estimating the total toxic potency of PAHs. Even though this TEF-based approach is relatively straight forward, it is not adequate for determining the total potency of real-life based complex mixtures of chemicals because it not feasible nor efficient to investigate every possible congener. As an example, in chapter 4, a complete analysis of the concentrations of all PAHs would have theoretically meant that the concentrations of over 500 PAHs reported in cigarette smoke had to be analysed (Rodgman and Perfetti, 2006), while for most of these congeners, information on their toxicity is not available. In addition, this TEF-based approach is also too dependent on prior knowledge of the presence of all chemicals in the sample that can activate a certain toxicity target and can therefore result in an underestimated risk of exposure.

In our opinion bioassays, like the DR_{human} and DR_{hp} CALUX are better equipped to the determination of the total toxic potency of complex mixtures than the traditional analytical chemistry-based methods. In fact, both DR_{human} and DR_{hp} CALUX bioassays were successfully applied to the quantitative analysis of complex AhR-active mixtures of dioxins (chapter 3, DR_{human} CALUX) and PAHs (chapter 4, DR_{hp} CALUX) originating from e.g., consumer goods (plastic toys) and in human tissues. In chapter 3, a good correlation was obtained between chemistry-determined total TEQ values and total bioassay-equivalent activity (BEQ). An even better correlation was obtained when comparing TEQ to total toxicity equivalent values calculated with bioassay congener-specific relative potency values (BEQ_{REP}). This additionally suggested that the observed differences between TEQ and BEQ in the toy sampled may be explained by the presence of compounds in the sampled different from the 17 PBDD/Fs investigated. In chapter 4, the same approach was employed (BEQ vs BEQ_{REP}) and the average total bioassay-equivalent PAH-mediated activities measured in the foetal livers were in line with BEQ_{REP} (using PAH-REP values from Pieterse et al., 2013) calculated from a study within the same cohort (Fowler et al., 2014). Moreover, in the future it would be relevant for environmental and human monitoring studies to first focus on measuring the total toxic potency of complex chemical mixtures in matrix extracts, using a range of reporter gene bioassays with different receptor-based modes of action. With the objective to examine the presence of potential hazards and thereafter confirm the presence of culprit chemicals using analytical chemistry-based methods.

2.4. Possible human health impact of observed effects using the newly developed AhR-based bioassays.

In this final paragraph, human exposure to AhR-active chemical mixtures as observed in chapter 3 (complex mixtures of PCDD/F and PBDD/F and other dioxin-like compounds present in children's toys made from recycled plastic) and chapter 4 (complex PAH-based foetal exposure related to smoking behaviour of pregnant women) are discussed in terms of their possible human health hazard consequences.

In chapter 3, we have estimated the possible intake of dioxins (in pg 2,3,7,8-TCDD-EQ/kg body weight/day) for human infants from the toys made of recycled plastic, based on information on child behaviour in relation to mouthing and WHO child growth standards (Ms Scientific Committee SCHER et al., 2016). This approach enables to roughly estimate if the exposure may pose a risk to young children and whether this exposure scenario warrants further attention and possible regulatory intervention. Results from this study implied that plastic toys may be an unexpected significant source of exposure to dioxin-like chemicals for children at a sensitive period of their development. It should be noted however that this estimation was hampered by, e.g., the lack of (human-relevant) exposure and toxicokinetic data for the chemicals studied in the exposure scenario investigated and leaching frequency/rates of chlorinated and brominated dioxins, and related dioxin-like compounds from plastic toys. Nevertheless, the fact that there is at present no attention to possible contamination of (particularly recycled) plastics and other polymers with unmanaged and toxic chemical mixtures, like brominated dioxins, indicate that the current methods in force to assess the safety of toys and other consumer goods are not sufficient to ensure that they do not pose a threat to human health. Future safety assessment studies should aim at investigating any source/type of material that is intended to be used in recycling processes to produce new-use or reuse in e.g. consumer goods. Therefore, these materials should be screened by adverse-outcome based reporter bioassays that can capture the total toxic potency of possible contaminants in raw materials, intermediates, and end-products; with the objective to inform on potential health hazard and the need of public health intervention by regulatory authorities.

In the study presented in chapter 4, the effect of maternal smoking on PAH-mediated AhR activity in the placenta and foetal liver was not estimated from a potential external source but directly measured in human tissue samples. The results of the analysis revealed that maternal smoking was associated with significantly higher levels of PAH-mediated activity in the placenta samples from smoking-mothers (4-fold) whereas surprisingly, no significant differences were observed between foetal liver samples from both groups, suggesting that the placenta might have operated somehow as a barrier to foetal exposure to (AhR interacting) PAHs. These results may have important implications for understanding the molecular basis for observed smoking-related adverse health effects in human infants, such as low birth weight (Choi et al., 2003; Perera Frederica et al., 2004) and neurobehavioral effects (Perera Frederica et al., 2012) and may emphasize that the AhR-signalling disruption *in utero* may play an important role in the prenatal initiation of (human) adverse effects.

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Summary

The aryl hydrocarbon receptor (AhR) is a major biological target of highly toxic chemicals (e.g., dioxins), and the presence of AhR-active chemicals in the environment can be associated with adverse outcomes in humans. Recent findings regarding the AhR indicate species-specific differences in ligand-specificity and sensitivity which are broader than initially thought. Such differences may have implications for human health-based hazard assessment, and it is expected that human toxic response to different classes of chemicals interacting with the AhR can be better predicted using human-based assays than widely used rodent-cell based assays. However, that aspect has not been fully investigated mainly because suitable human-based assays are lacking. In this thesis, a human cell-based CALUX bioassay was generated and its applicability towards hazard assessment of chemicals and mixtures related to human-health, was evaluated in comparison to rodent-based CALUX cell lines.

Chapter 1 gives a general introduction to concepts and matters covered in the thesis. The chapter provides background on mixture toxicology and its challenges with regards to human health. Next, the AhR functions, main groups of ligands, and the notion of AhR-mediated toxicity are defined because of the major role the receptor played throughout the thesis. Then, the traditional and alternative approaches for hazard assessment of the chemicals investigated in this thesis (dioxins, PAHs, and mycotoxins) are described. Finally, the principle of cell-based bioassays for the analysis of AhR-mediated activity is presented and its main applications described.

The historical emphasis in the development of AhR-mediated bioassays was to obtain the highest sensitivity for the quantification of dioxin-like chemicals in e.g., food samples. Therefore, cell lines derived from the most dioxin-sensitive species (e.g., rodents) were used to design bioassays. However, this resulted in a lesser focus on developing efficient human-relevant AhR-reporter gene assays to investigate the effect of chemicals and mixtures on the AhR in a human cellular context with human hazard assessment purposes. Therefore, the first step in this thesis was to develop a human-based AhR-mediated CALUX bioassay. **Chapter 2** describes the development and characterization of the DR_{human} CALUX, a novel human-relevant HepG2-based AhR-based luciferase reporter gene assay. In this study, the bioassay is applied to determine the potency of 21 chlorinated (PCDD/Fs) and brominated (PBDD/FS) dioxins to activate the human AhR. Then, we compared the potency values obtained in the DR_{human} CALUX with values obtained with the rat cell-based bioassay (DR CALUX). The results indicate that PBDD/Fs, like PCDD/Fs, are potent activators of both human and rodent Ah-receptor signalling pathways. However, rodent cells appear to be more sensitive to dioxins in terms of absolute sensitivity, with a typical 10-100-fold difference with the DR_{human} CALUX based on the EC_{50} of the analysed dioxins. A final comparison of the relative potency values determined in the DR_{human} CALUX with the WHO toxic equivalency factors (TEF) values for human hazard assessment was made. The results indicate that the interim TEFs

proposed for PBDD/Fs are in line with their potency determined in the DR_{human} CALUX cells whereas some deviations are observed for PCDD/Fs.

In **chapter 3** the DR_{human} CALUX bioassay is applied to determine total dioxin-like activity in samples of consumer goods, here plastic toys. These toys are suspected to contain high levels of legacy brominated dioxins (PBDD/Fs) originating from brominated flame-retardant precursors also present in some plastics. An analytical chemistry-based analysis (GC-HRMS) indicated that brominated dioxins (PBDD/Fs) were present in all samples and toxicity-equivalent activity (TEQ) values were calculated and compared to total bioassay-based dioxin-like activity (BEQ) determined using DR and DR_{human} CALUX. Overall, a good correlation is observed between analytical chemistry determined TEQ and bioassay BEQ. Finally, an estimate of exposure based on the ingestion of contaminated plastic from toys by young children through mouthing behaviour was conducted. Based on DR_{human} CALUX activity, none of the exposures assessed for the plastic toys is below the tolerable dietary intake for dioxin-like compounds. This chapter shows the relevance of using the DR_{human} CALUX bioassay for human health-based hazard assessment of mixtures of chlorinated, brominated, and related AhR active contaminants.

The DR_{human} provides a human cellular context that is desirable to reach a higher degree of human relevance and a more adequate toxicological risk assessment. However, it is less sensitive than rodent-based assays (e.g. DR CALUX). Hence, less suitable for the sensitive analysis of human samples in biomonitoring and epidemiological studies which often combine a small amount of tissue with low concentrations of AhR active compounds. Even then, in certain cases, the bioassay analysis of small sample volumes (e.g. foetal samples) may only be feasible by using even more sensitive methods than those available now. **Chapter 4** describes the development of such a method, a highly sensitive high performance (hp) variant of the rodent-based DR CALUX bioassay, the DR_{hp} CALUX. The bioassay shows improved sensitivity compared to existing AhR-responsive CALUX bioassays, particularly towards polycyclic aromatic hydrocarbons (PAHs). In this study, in collaboration with the University of Aberdeen (partner from the EU-ProtectED network), the DR_{hp} CALUX bioassay was applied to analyse PAH-mediated AhR activity in placentas and human foetal liver samples to investigate the effect of maternal smoking on placental and foetal hepatic AhR activity. The optimized DR_{hp} CALUX bioassay is able to detect and quantify total AhR-based activity of PAHs expressed in B[a]P-equivalent activity in small samples (200mg) of human tissue, with a limit of detection of 7 pg of B[a]P-equivalent activity per gram of tissue sample. The main finding of this study is that maternal smoking resulted in a significant increase in PAH-mediated AhR activity (4-fold) in the placenta of smoking mothers as compared to non-smoking mothers. There was no significant difference between foetal livers from non-smoking and smoking mothers. The results of this study suggest that the levels of PAHs present in the placenta of smoking mothers may affect normal AhR-signalling and hence, with possible implications for developmental outcomes in exposed foetuses, such as reduced birth weight and cognitive development.

Rodent-based assays, such as the DR_{hp} CALUX, are suitable for highly sensitive detection of AhR-activity in samples. However, in studies investigating the toxicity mechanism of a chemical, (organ-) species-specific bioassays are more adequate. Indeed, the use of species-specific can significantly enhance the predictability and relevance of suspected effects in humans by providing a more accurate view of the sequence of molecular events leading to toxic effects. This is illustrated in **chapter 5**, where the role of the AhR in the human hepatic toxicity of mycotoxins is studied. In this study, we collaborated with the TOXALIM research centre in food Toxicology from the French national institute of agricultural research (INRAe, another partner from the EU-ProtectED network). The focus of this study was on aflatoxin B1 and its precursor versicolorin A, and the aim to investigate the possible role of AhR-mediated signalling in aflatoxins genotoxicity. Human liver relevant *in vitro* bioassays were used as proxies for human genotoxicity as well as the DR_{human} CALUX and CYP450s gene expression studies. The results show that both mycotoxins are cyto- and genotoxic and that versicolorin A, but not aflatoxin B1, was capable to induce AhR-transactivation in a dose-dependent manner. The AhR induction by versicolorin A was associated with CYP450 induction, thus suggesting that the mycotoxin can influence aflatoxin B1 genotoxicity by promoting its bioactivation by CYP450s to a highly DNA-reactive and genotoxic exo-AFB1-8, 9-epoxide. The combination of versicolorin A with aflatoxin B1 resulted in enhanced cyto- and genotoxic effects, supporting the hypothesis that AhR activation promoted by versicolorin A is likely associated with a greater bioactivation of aflatoxin B1. The results of this study are relevant for risk assessment of aflatoxin toxicity related to food exposure and also show the applicability of human-based reporter cell lines in hazard identification and food safety studies.

In **chapter 6**, the results obtained in the different studies are summarized and conclusions drawn. Additionally, an outlook is presented on the relevance of the obtained results and developed methods for future human health-based risk assessment and monitoring studies.

Samenvatting

De arylkoolwaterstofreceptor (AhR) is een belangrijk biologisch doeleiwit (receptor) voor zeer toxische stoffen (o.a. dioxines). De aanwezigheid van AhR actieve stoffen in het milieu kan worden geassocieerd met negatieve gezondheidseffecten bij de mens. Recente bevindingen aangaande de AhR geven aan dat er grotere verschillen bestaan in ligand-specificiteit en in gevoeligheid tussen diersoorten, dan eerder werd gedacht. Dergelijke verschillen kunnen mogelijk gevolgen hebben voor het afschatten van gezondheidsrisico's voor de mens. Derhalve wordt verondersteld dat humane cellijn-gebaseerde meetmethoden een betere voorspelling zouden kunnen geven van de te verwachten toxiciteit van giftige stoffen voor de mens, dan degene die zijn gebaseerd op de breed-toegepaste knaagdier-cellijnen. Echter, tot voorkort was dit aspect nog niet voldoende onderzocht, vanwege het afwezig zijn van, of tekort aan bruikbare en geschikte humane cellijn-gebaseerde bioassays. In dit proefschrift zijn nieuwe luminescentie-gebaseerde humane AhR reporter-cellijnen (CALUX) geconstrueerd en onderzocht op hun toepasbaarheid voor het meten en evalueren van effecten van (mengsels) van chemische stoffen op de menselijke gezondheid in vergelijking tot reeds bestaande knaagdier-gebaseerde cellijnen.

Hoofdstuk 1 beschrijft een algemene introductie van de concepten en onderzoeken uitgevoerd en beschreven in dit proefschrift. In dit hoofdstuk wordt achtergrondinformatie gegeven over (mengsel)toxicologie en de uitdagingen hiervan bij het bepalen van humane gezondheidseffecten. Verder wordt de Ah-receptor geïntroduceerd en uitgelegd welke belangrijke mechanistische rol de AhR speelt in de toxiciteit van chemische stoffen, inclusief een beschrijving van de belangrijkste groepen aan AhR-liganden. De onderzoeken in dit proefschrift zijn allemaal gericht op de mogelijke rol van c.q., de werking via de AhR van enkele voorbeeldstofgroepen. Verder worden traditionele en alternatieve methodes voor bepaling van de giftigheid (hazard assessment) geïntroduceerd in dit proefschrift voor de stofgroepen: dioxinen, polycyclische aromatische koolwaterstoffen, en mycotoxinen. Tot slot worden de principes van cel-gebaseerd bioassays voor de analyse van AhR gemedieerde activiteit beschreven inclusief de belangrijkste toepassingen hiervan.

Bij het ontwikkelen van AhR-gemedieerde bioassays werd tot voor kort primair de nadruk gelegd op het bereiken van een zo hoog mogelijke gevoeligheid om hiermee de vaak extreem lage concentraties aan dioxinen in e.g., levensmiddelen te kunnen kwantificeren. Hiervoor werden cellijnen van de meest dioxinegevoelige soorten (v.b. knaagdieren) gebruikt als basis voor het ontwerpen van gevoelige bioassays. Echter, hierdoor was er minder focus op het ontwikkelen van AhR-gebaseerde bioassays met het beste voorspellende vermogen voor effecten bij de mens, ten behoeve van de humane risicoschatting. Daarom was de eerste stap in het promotieonderzoek beschreven in dit proefschrift het ontwikkelen van een op menselijke cellijn-gebaseerde AhR CALUX bioassay.

Hoofdstuk 2 beschrijft de ontwikkeling en karakterisering van de DR_{human} CALUX, een nieuwe op de levertumor cellijn HepG2-gebaseerde AhR-luciferase reporter-gen bioassay met naar verwachting hoge relevantie voor humane risicoschatting van toxische stoffen. Deze nieuw ontwikkelde bioassay is na het doorstaan van een validatie-fase gebruikt voor het bepalen van de AhR-gemedieerde luciferase inductie potentie van 21 gechloreerde (PCDD/Fs) en gebromeerde (PBDD/Fs) dioxine congeneren. Daarna werden de relatieve potentiewaarden bepaald in de DR_{human} CALUX vergeleken met de behaalde potentiewaarden in de welbekende en veel gebruikte ratten H4IIe levercel-gebaseerde bioassay (DR CALUX). De resultaten geven aan dat PBDD/Fs net als PCDD/F congeneren, sterke activators zijn van de AhR-signaleringsroute in zowel de ratten- als in de menselijke cellijnen. Echter, de gevoeligheid van knaagdiercellen (DR CALUX) voor dioxinen blijkt in absolute zin ruim hoger te zijn, een factor 10-100 verschil in EC_{50} waarden, in vergelijking tot de gevoeligheid van DR_{human} CALUX bioassay, i.e., een waarneming die past bij de notie dat de mens (veel) minder gevoelig is voor de (lever)toxische effecten van dioxinen, dan de rat. Als laatste zijn de relatieve potentiewaarden zoals bepaald in de DR_{human} CALUX vergeleken met de door de WHO vastgestelde toxische equivalentie factoren (TEF) die gebruikt worden bij humane risicoschatting. Deze vergelijking laat zien dat de interim TEFs voorgesteld door de WHO voor de gebromeerde dioxinen en furanen (PBDD/Fs) vergelijkbaar zijn met de potentiewaarden bepaald in de DR_{human} CALUX cellen, terwijl er voor de gechloreerde PCDD/Fs enige verschillen werden gevonden.

In **hoofdstuk 3** is de DR_{human} CALUX assay toegepast om de totale dioxineachtige activiteit te kunnen bepalen in monsters van consumentengoederen, in dit geval plastic speelgoed. Dit uit gerecycled afvalplastic gemaakt speelgoed wordt ervan verdacht dat het relatief hoge concentraties aan gebromeerde dioxinen (PBDD/Fs) kan bevatten, die ontstaan zijn uit broomhoudende precursors die als brandvertragers zijn toegevoegd aan bepaalde plastics. Analyse van monsters van dit plastic speelgoed, via een analytisch-chemische methode, gaschromatografie-gekoppeld aan hoge resolutie massa spectrometrie (GC-HRMS), toonde aan dat relatief hoge gehaltes aan gebromeerde dioxinen (PBDD/Fs) aanwezig waren in alle gemeten monsters. Hierna werden dioxine toxiciteits-equivalenten (TEQ) berekend en deze werden vergeleken met de totale dioxin-like (BEQ) waarden gemeten met behulp van DR CALUX en DR_{human} CALUX bioassays. Over het algemeen werd een goede correlatie gevonden tussen de analytisch-chemisch bepaalde en berekende TEQ en de via bioassay bepaalde BEQ waarden. Tot slot is er een schatting gemaakt van de blootstelling aan dioxinen bij jonge kinderen via het inslikken van vervuild plastic uit speelgoed, via zogenaamde “mouthing behavior” van kinderen. Uit de gemaakte afschattingen uit speelgoed en de daarmee geassocieerde verwachte extra dioxine-inname bleek dat de verwachte blootstelling aan dioxinen via mouthing behavior van vervuild plastic, gebaseerd op de DR_{human} CALUX activiteitmetingen, in alle gevallen leidde tot een hogere inname dan de tolereerbare dagelijkse inname (TDI) norm voor dioxineachtige stoffen. Dit hoofdstuk toont de relevantie van het gebruik van

DR_{human} CALUX bioassays voor schatting van gezondheidsrisico’s van mengsels aan gechloreerde, gebromeerde dioxinen en overige AhR actieve contaminanten.

De DR_{human} CALUX cellijn levert de humane context die wenselijk is om een hoger niveau aan menselijke relevantie en een adequatere toxicologische risicobeoordeling te bereiken. Echter, de DR_{human} CALUX cellijn is minder gevoelig voor dioxinen dan de op knaagdier gebaseerde assays (v.b. DR CALUX) en is daardoor minder geschikt voor het inzetten als gevoelig meetinstrument voor lage hoeveelheden aan dioxinen in bij voordbeeld voedingsmiddelen en in menselijke samples, met vaak geringe samplevolumes uit b.v. epidemiologische studies. In sommige situaties beschikken we over zo weinig monstervolume i.e., foetaal weefselmateriaal dat nog gevoeligere versies van AhR-gebaseerde bioassays nodig zijn.

Hoofdstuk 4 beschrijft de ontwikkeling van een AhR-gebaseerde bioassay met de hoogst mogelijke gevoeligheid, een zogenaamde high performance (hp) variant van de rattenlevercel gebaseerde DR CALUX assay, genaamd DR_{hp} CALUX. Deze DR_{hp} CALUX toont een hogere gevoeligheid voor met name polycyclische aromatische koolwaterstoffen (PAHs) dan de reeds beschikbare AhR-responsieve CALUX bioassays. Deze DR_{hp}-CALUX cellijn is toegepast in een studie die in samenwerking met de University of Aberdeen (partner van de EU-ProtectED netwerk) is uitgevoerd. Hierbij werd de totale PAH-gemedieerde AhR activiteit bepaald in kleine hoeveelheden van humaan placenta en foetaal materiaal. Deze samples zijn met toestemming van de medisch-ethische commissie genomen door de University of Aberdeen en zijn afkomstig van vrijwillig afgebroken zwangerschappen. Het doel van dit onderzoek was om de mogelijke nadelige gezondheidseffecten van roken tijdens de zwangerschap te bepalen, door ondermeer de AhR activiteit te meten in monsters van de placenta en foetus. De geoptimaliseerde DR_{hp} CALUX bioassay bleek in staat om de totale AhR-gemedieerde activiteit van PAHs, uitgedrukt in B[a]P-equivalenten (BaP-EQ) in zeer kleine monsters (200mg) van menselijk weefsel te detecteren en te kwantificeren, met een detectie limiet van 7 pg of B[a]P-equivalent activiteit per gram weefsel. De voornaamste waarneming uit deze studie was dat roken tijdens de zwangerschap een verhoging van PAH gemedieerde AhR activiteit (4 keer hoger) in de placenta tot gevolg heeft van rokende zwangeren vergeleken met niet-rokende zwangeren. Er was geen significant verschil in PAH activiteit waar te nemen in foetale levermonsters van rokende en niet-rokende moeders. Deze resultaten suggereren dat de waargenomen bioactiviteit van PAHs in de placenta effect kan hebben op normale AhR signalering en daardoor mogelijke implicaties kan hebben voor de ontwikkeling van blootgestelde foetussen, zoals een verminderd geboortegewicht en vertraagde cognitieve ontwikkeling.

Uit deze resultaten blijkt dat rattenlevercel-gebaseerde AhR-bioassays zoals de DR_{hp} CALUX zeer bruikbaar zijn indien hoge gevoeligheid nodig is gecombineerd met geringe samplevolumes, zoals in klinisch-epidemiologische studies. In studies naar het werkingsmechanisme van een chemische stof zijn soort-specifieke en orgaan-specifieke

bioassays nuttig vanwege de te verwachten hogere voorspelbaarheid en relevantie van de bioassayresultaten met betrekking tot nadelige gezondheidseffecten bij de mens.

In **hoofdstuk 5** hebben we gebruikmakend van een combinatie van humane cel-gebaseerde bioassays gepoogd om de volgorde van belangrijke moleculaire gebeurtenissen te volgen die uiteindelijk zouden kunnen leiden to orgaan-specifieke toxiciteit. Een toepassingsvoorbeeld hiervan is beschreven in dit hoofdstuk waarbij de rol van de AhR in hepatisch toxiciteit van mycotoxinen in een menselijke cellulaire context is bestudeerd. Tijdens deze studie is samengewerkt met het TOXALIM onderzoekscentrum in Food Toxicology van het Franse Nationale Instituut voor Landbouwkundig Onderzoek (INRA), een andere partner uit EU-ProtectED netwerk. De focus van dit onderzoek was gericht op aflatoxine B1 en zijn precursor versicolorine A en had als doel om de mogelijke rol van AhR gemedieerde signalering in de genotoxiciteit van aflatoxine te onderzoeken. Menselijke levercel-gebaseerde *in vitro* bioassays werden gebruikt as proxy voor menselijke genotoxiciteit inclusief humane metabole aspecten, o.m. gebruikmakend van DR_{human} CALUX en CYP450s genexpressiestudies. De resultaten laten zien dat beide mycotoxinen cyto- en genotoxisch zijn en dat versicolorine A, maar niet aflatoxine B1, in staat was tot dosis-afhankelijke AhR transactivatie. De AhR inductie door versicolorine A was geassocieerd met een significante toename in CYP450 enzymexpressie. Hierdoor is het aannemelijk dat de precursor, versicolorine A, de concentratie aan zeer DNA reactieve en genotoxische exo-AFB1-8, 9-epoxide metabolieten, die gevormd worden via omzetting door CYP450 enzymen, sterk kan verhogen, waardoor een combinatieblootstelling aan versicolorine A met aflatoxine B1 resulteerde in versterkte cyto- en genotoxische effecten. Deze resultaten ondersteunen de hypothese dat AhR activatie door versicolorine A waarschijnlijk is geassocieerd met een grotere bio-activatie van aflatoxine B1 tot reactieve en genotoxische metabolieten. De resultaten van deze studie zijn relevant voor risicobeoordeling van aflatoxinen in voedsel en toont de toepasbaarheid van humane cel-gebaseerde reportercellijnen bij de identificatie en risicobeoordeling van mengsels van aflatoxinen en gerelateerde stoffen.

In **hoofdstuk 6** worden de resultaten behaald in de verschillende onderzoeken samengevat, bediscussieerd en worden conclusies getrokken. Ook wordt er een vooruitblik geschetst op de relevantie van de behaalde resultaten en van de ontwikkelde methodes voor toekomstige, op de menselijke gezondheid toegespitste risicobeoordelings- en monitoringstudies.

Résumé

Le récepteur d'aryle hydrocarbone (AhR) est une importante cible biologique pour certains composés chimiques extrêmement toxiques (e.g. les dioxines). La présence de composés capable d'interagir avec le AhR dans l'environnement peut être associée à des effets néfastes pour l'humain. Des découvertes récentes concernant l'AhR indiquent des différences spécifiques aux espèces en termes de spécificité et de sensibilité aux ligands, qui sont plus importantes qu'on ne le pensait initialement. Ces différences peuvent avoir des implications pour l'évaluation des risques pour la santé humaine. De ce fait, il est attendu que la réponse toxique de l'humain à différentes classes de composés interagissant avec le AhR puisse être mieux prédite en utilisant des bio-essais basés sur des cellules humaines plutôt que bio-essais basés sur des cellules de rongeurs, qui sont pourtant largement utilisés. Toutefois, cet aspect n'a pas été pleinement étudié, principalement en raison de l'absence de bio-essais appropriés pour représenter un contexte cellulaire humain. Dans cette thèse, un bio-essai CALUX basé sur des cellules humaines a été généré. Son applicabilité à l'évaluation des dangers de composés et mélange de composés en rapport à la santé humaine a été évaluée en comparaison avec des bio-essais CALUX basés sur des cellules de rongeurs.

Le **premier chapitre** fournit une introduction générale aux concepts et sujets couverts dans la thèse. Ce chapitre apporte des informations générales sur la toxicologie des mélanges ainsi que les défis à relever par la toxicologie en matière de santé humaine. Ensuite, les fonctions de l'AhR, ses principaux groupes de ligands, ainsi que la notion de réponse toxique médiée par le AhR sont définis en raison du rôle majeur joué par le récepteur tout au long de la thèse. Par la suite, les approches traditionnelles et alternatives pour l'évaluation des dangers des composés étudiés dans cette thèse (dioxines, hydrocarbures aromatiques polycycliques et mycotoxines) sont décrites. Enfin, le principe des bio-essais cellulaires pour l'analyse de l'activité médiée par le AhR est présenté et ses principales applications sont décrites.

Historiquement, la priorité dans le développement de bio-essais permettant d'analyser l'activité médiée par le AhR a été de d'obtenir la meilleure sensibilité possible afin de quantifier des composés analogues de dioxines, par exemple dans les denrées alimentaires. C'est pourquoi des lignées de cellules provenant des espèces les plus sensibles aux dioxines (e.g. les rongeurs) ont été privilégiées pour développer des bio-essais. Cependant, cela a eu pour effet de réduire l'importance accordée au développement de bio-essais basés sur des lignées cellulaire humaines afin d'étudier l'effet de (mélange de) composés sur l'AhR dans un contexte cellulaire humain dans le but d'évaluer les risques pour la santé humaine. Par conséquent, la première étape dans cette thèse a été de mettre au point un test CALUX à médiation AhR basé sur des cellules humaines. Le **chapitre 2** décrit le développement et la caractérisation du DR_{human} CALUX, un nouveau bio-essai basé sur des cellules humaines hépatiques exprimant un gène rapporteur de luciférase induit par l'activation du AhR. Dans cette étude, le bio-essai est

appliqué pour déterminer la capacité de 21 dioxines chlorées (PCDD/Fs) et bromées (PBDD/Fs) à activer l'AhR. Nous avons ensuite comparé les potentiels d'induction obtenus dans les cellules humaines avec les résultats obtenus avec le bio-essai basé sur des cellules de rat DR CALUX. Les résultats montrent que les PBDD/Fs, comme les PCDD/Fs, sont de puissants activateurs des voies de signalisation de l'AhR chez l'Homme et le rat. Cependant, les cellules de rats s'avèrent plus sensibles aux dioxines en termes concentration absolue (10 à 100 fois plus sensibles) basé sur l'EC_{50} des dioxines testées. Ensuite, les potentiels d'induction des dioxines obtenus avec le DR_{human} CALUX sont comparés avec les valeurs des facteurs d'équivalence toxique (TEF) de l'OMS utilisées pour l'évaluation des dangers pour l'homme. Les résultats indiquent que les TEF provisoires proposées pour les PBDD/Fs sont conformes à leur potentiel déterminé dans les cellules humaines, alors que des écarts sont observés pour les PCDD/Fs.

Dans le **chapitre 3** nous appliquons ensuite ce nouveau bio-essai pour quantifier des composés à activité analogue à la dioxine dans des biens de consommation, ici des jouets en plastique. En effet, certains jouets en plastique sont soupçonnés de contenir des niveaux élevés de PBDD/Fs provenant de retardateurs de flammes bromés précurseurs qui peuvent être présents dans certains plastiques. Premièrement, une analyse chimique (CG-SMHR) a indiqué que des PBDD/Fs étaient présentes dans tous les échantillons. L'activité toxique équivalente (TEQ) dans les échantillons a été calculée et comparée à l'activité biologique totale déterminée par les bio-essais DR et DR_{human} CALUX (BEQ). Dans l'ensemble, une forte corrélation est observée entre la TEQ déterminée par la chimie analytique et la BEQ déterminée par les bio-essais. Montrant ainsi qu'il est possible d'utiliser le DR_{human} CALUX pour l'analyse de mixtures complexes et la quantification d'analogues de dioxines. Enfin, une estimation d'exposition aux PBDD/Fs basée sur l'ingestion de plastique contaminés via la mastication des jouets par des enfants en bas âge est réalisée. Sur la base de l'activité BEQ obtenue avec le DR_{human} CALUX, aucune des expositions évaluées n'est inférieure à la dose alimentaire tolérable pour les composés analogue de dioxines. Ce chapitre montre la pertinence de l'utilisation du DR_{human} CALUX pour l'évaluation des risques pour la santé humaine de mélanges de contaminants chlorés, bromés et autres contaminants capables d'activer l'AhR.

Le DR_{human} fournit un contexte cellulaire humain qui est souhaitable pour atteindre un degré plus élevé de pertinence pour l'humain et une évaluation plus adéquate des risques toxicologiques. Néanmoins, le DR_{human} CALUX est moins sensible que les bio-essais développés dans des cellules de rongeurs et de ce fait est moins adapté à la quantification d'activité médiée par le AhR dans des échantillons humains. Par exemple dans le cadre d'études de biosurveillance et d'études épidémiologiques qui combinent souvent une petite quantité de tissu avec de faibles niveaux d'activité médiée par le AhR. Cependant, l'analyse de petits volumes d'échantillons (par exemple, des échantillons venant de fœtus) ne peut être réalisée qu'en utilisant des méthodes encore plus sensibles. Même dans ce cas, les bio-essais disponibles peuvent ne pas être assez sensible et il est nécessaire de développer de nouveaux bio-essais. Le **chapitre 4** décrit le développement d'un tel bio-essai, une variante hautement sensible et plus performante du DR CALUX, le

DR_{hp} CALUX, qui est également basé sur des cellules de rats. Le DR_{hp} CALUX s'avère plus sensible que les autres bio-essais CALUX quantifiant l'activation du AhR, en particulier vis-à-vis des hydrocarbures aromatiques polycycliques (HAP). Dans cette étude, en collaboration avec l'Université d'Aberdeen (partenaire du réseau EU-ProtectED), le DR_{hp} CALUX est appliqué pour analyser l'activité AhR induite par les HAP dans des placentas et échantillons de foie de fœtus humain afin d'étudier l'effet du tabagisme maternel sur l'activité AhR placentaire et hépatique du fœtus. Une fois optimisé, le DR_{hp} CALUX a permis de détecter et de quantifier l'activité AhR totale induite par les HAPs exprimée en activité équivalente au B[a]P dans de petits échantillons (200 mg) de tissu humain. Ce avec une limite de détection de 7 pg d'activité équivalente au B[a]P par gramme d'échantillon de tissu. La principale conclusion de cette étude est que le tabagisme maternel entraîne une augmentation significative de l'activité AhR dans le placenta des mères fumeuses par rapport aux mères non fumeuses (4 fois supérieure). Il n'y avait pas de différence significative entre les foies des fœtus de mères non-fumeuses et des mères fumeuses. Les résultats présentés dans ce chapitre suggèrent que les niveaux de HAP présents dans le placenta des mères fumeuses peuvent affecter la voie de signalisation AhR, avec de possibles implications pour le développement des fœtus exposés, tels que la réduction du poids de naissance et des retards de développement cognitif.

Les bio-essais basés sur des cellules de rongeurs, tels que le DR_{hp} CALUX, sont particulièrement adaptés à la détection de très petites quantités de composés capable d'activer le AhR dans des échantillons complexes. En revanche, pour des études visant à étudier le mécanisme de toxicité d'un composé, des bio-essais spécifiques à l'espèce (et organe) étudiée sont plus adéquats et peuvent considérablement améliorer la prévisibilité et la pertinence des effets suspectés chez l'Homme. Cela en fournissant une vision plus représentative de la séquence des événements moléculaires conduisant à des effets toxiques. Ceci est illustré dans le **chapitre 5**, où le rôle de l'AhR dans la génotoxicité hépatique humaine des mycotoxines est étudié. Dans cette étude, nous avons collaboré avec le centre de recherche en Toxicologie alimentaire TOXALIM de l'Institut National français de Recherche Agronomique (INRAe, autre partenaire du réseau EU-ProtectED). Cette étude porte sur l'aflatoxine B1 et son précurseur, la versicolorine A, et a pour but d'étudier le rôle possible de la voie de signalisation de l'AhR dans la génotoxicité des aflatoxines. Des bio-essais représentatifs du foie humain *in vitro* ont été utilisés comme modèles de génotoxicité humaine, ainsi que le DR_{human} CALUX et des études d'expression des cytochromes P450 (CYP450) dans les cellules HepG2. Les deux mycotoxines se sont avérées cyto- et génotoxiques. La versicolorine A, mais pas l'aflatoxine B1, était capable d'activer le AhR de manière dose-dépendante. L'induction de l'AhR est associée à l'induction des CYP450s, ce qui suggère que la versicolorine A peut influencer la génotoxicité de l'aflatoxine B1 en favorisant sa bioactivation par les CYP450s en exo-AFB1-8, 9-époxyde capable de réagir avec l'ADN et de provoquer des lésions. La combinaison de la versicolorine A avec l'aflatoxine B1 a entraîné des effets cyto- et génotoxiques accrus, ce qui soutient l'hypothèse selon laquelle l'activation des AhR favorisée par la versicolorine A est probablement associée à une bioactivation plus

importante de l'aflatoxine B1. Les résultats de cette étude sont importants pour l'évaluation des risques de toxicité des aflatoxines liés à l'exposition via l'alimentation. Ils montrent également l'applicabilité et l'utilité de bio-essais développés dans des cellules humaines pour l'identification des dangers et dans des études de sécurité alimentaire par exemple.

Enfin dans le **chapitre 6**, les résultats obtenus dans les différentes études présentées sont résumés et des conclusions sont tirées. De plus, un bilan de la pertinence des résultats obtenus, ainsi que des méthodes développées pour de futures études d'évaluation et de surveillance des risques pour la santé humaine est présenté.

List of publications

Budin, C., Besselink, H., van Vugt-Lussenburg, B.M.A., Man, H.-Y., van der Burg, B., Brouwer, A., 2021. Induction of AhR transactivation by PBDD/Fs and PCDD/Fs using a novel human-relevant, high-throughput DRhuman CALUX reporter gene assay. Chemosphere 263, 128086.

Budin, C., Petrlik, J., Strakova, J., Hamm, S., Beeler, B., Behnisch, P., Besselink, H., van der Burg, B., Brouwer, A., 2020. Detection of high PBDD/Fs levels and dioxin-like activity in toys using a combination of GC-HRMS, rat-based and human-based DR CALUX® reporter gene assays. Chemosphere 251, 126579.